STUDIES ON TWO ABNORMAL HUMAN ALBUMINS, ALBUMINS REDHILL AND WARWICK-2

by

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A thesis submitted in partial
fulfilment of the requirements
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Sciences

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To Mum and Dad

with love

Nothing's impossible, I have found,
For when your chin is on the ground,
You pick yourself up, dust yourself off,
Start all over again.

Jerome Kern, 1936

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ABSTRACT

Albumins Redhill and Warwick-2 are monomeric, slow albumin variants discovered serendipitously in sera obtained from two unrelated families living in the U.K.

Albumin Redhill was purified both by chromatofocusing and preparative polyacrylamide gel electrophoresis whereas albumin Warwick-2 was obtained exclusively by the latter technique. Following isolation, selective cleavage at methionine and tryptophan residues using specific chemical reagents was employed to examine the primary structure of each variant compared to the structure of normal albumin.

Albumin Redhill is a proalbumin; the intracellular precursor of albumin. An amino acid substitution within the N-terminal propeptide has prevented normal cleavage of the additional residues *in vivo*. A second mutation lies in the C-terminal region of the sequence, probably between residues 503 and 585. This mutation is unusual in that it appears to involve the insertion of a short sequence, corresponding to approximately ten to twelve amino acids. The N-terminal residue is L-arginine; L-leucine was identified as the C-terminal end group.

Albumin Warwick-2 contains an amino acid substitution in the N-terminal region of the sequence, between residues 1 and 87. This variant is not a proalbumin, having L-aspartic acid in the N-terminal position. The C-terminal residue is L-leucine.

The isoelectric point of albumin Redhill is more acidic than that of albumin A, at pH 4.74-4.75, whereas albumin Warwick-2 becomes isoelectric on the basic side of albumin A, at pH 5.0-5.02.

Both variants share a common antigenicity with albumin A and are present in a lesser quantity than the normal allotype in the serum. Albumin Redhill shows a reduced binding capacity for copper$^{(II)}$ and nickel$^{(II)}$ while albumin Warwick-2 binds these two metals normally. Both albumins show decreased binding for bilirubin and differential binding towards a series of organic dyes, but bind palmitate similarly to albumin A.

DECLARATION

The work described herein was carried out in the Department of Chemistry and Molecular Sciences at the University of Warwick, between October 1980 and September 1983, and was funded by the Science and Engineering Research Council. It is the original work of the author, except where specific acknowledgement is made or implied. This thesis has not been submitted for any other degree previously.

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Above all, I should like to thank my supervisor, Dr. D. W. Hutchinson, for his encouragement and advice throughout the course of this project.

***

PUBLICATIONS

1. Albumin Redhill, a human albumin variant.
   S. Brand, D. W. Hutchinson and D. Donaldson
   Submitted to *Clin. Chim. Acta* (1983)

2. Albumins Warwick-1 and Warwick-2, two human albumin variants.
   H. Y. N. Au, S. Brand, D. W. Hutchinson and P. Matejtschuk
   Submitted to *I.R.C.S. Med. Sci.* (1983)

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CHAPTER 1

NORMAL SERUM ALBUMIN

1.1 FUNCTIONS OF ALBUMIN

Albumin is a versatile, multifunctional protein important in many aspects of physiology, chemistry and clinical diagnosis. It is the most abundant protein in the blood, constituting approximately 60% of the total plasma protein and is normally present at a concentration of 42 g/litre, although this value may fluctuate during disease.

One of the main functions is to bind large organic anions normally insoluble in aqueous fluids and transport them to the liver for detoxification. These include drugs, dyes, fatty acids, metabolites, steroid hormones, bilirubin, organic toxins, tryptophan and heavy metal ions. Albumin also acts as an overflow depot for thyroxine and cortisol when the capacities of their more specific binding proteins, thyroxine - and cortisol binding globulin are exceeded.

Since albumin is too large to be lost extensively through the capillaries, a second major physiological role is the maintenance of plasma colloidal osmotic pressure, of which it provides 80% and hence contributes to the regulation of fluid exchange in the tissues. In addition, breakdown into amino acids provides nutrition for the peripheral tissues.

Owing to its high stability and solubility, albumin is often used as a model protein for protein chemistry and metabolic studies, and in immunology as a model antigen and hapten carrier. Since it is one of the few readily available carbohydrate-free proteins, albumin is widely used as a reference standard in protein assays.

For clinicians, blood albumin concentration is of greater diagnostic value than the measurement of total plasma protein. Albumin levels fall during disease: oedema, heart attacks, cirrhosis, nephritis, bronchitis, haemorrhage and gastrointestinal disease, or is lost through the skin after surgery, burns, dermatitis and accidental injury. Albumin levels are especially valuable as an index of the severity of protein malnutrition in children.

1.2 THE STRUCTURE OF SERUM ALBUMIN

1.2.1 The Primary Structure of Human Serum Albumin (HSA)

The albumin molecule consists of a single polypeptide chain comprising 585 amino acid residues. The complete primary sequence has been determined by Behrens *et al.*(1975) and Meloun *et al.*(1975) using conventional protein sequencing methods. There are several discrepancies between the two sequences, generally involving the structure around cysteine

```
                                                      -18          p r e           -10
                                                      met lys trp val thr phe ile ser leu leu phe leu phe ser
        (A)GCTTTTCTCTTCTGTCAACCCCACACGCCTTTGGCACA ATG AAG TGG GTA ACC TTT ATT TCC CTT CTT TTT CTC TTT AGC  (80)

        -1  -6    p r o    -1  1                               10                                      20
ser ala tyr ser arg gly val phe arg arg asp ala his lys ser glu val ala his arg phe lys asp leu gly glu glu asn phe lys
TCG GCT TAT TCC AGG GGT GTG TTT CGT CGA GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG TTT AAA GAT TTG GGA GAA GAA AAT TTC AAA (170)

21                                30          34                  40                                      50
ala leu val leu ile ala phe ala gln tyr leu gln gln cys pro phe glu asp his val lys leu val asn glu val thr glu phe ala
GCC TTG GTG TTG ATT GCC TTT GCT CAG TAT CTT CAG CAG TGT CCA TTT GAA GAT CAT GTA AAA TTA GTG AAT GAA GTA ACT GAA TTT GCA (260)

51      53                        60      62                      70                  75                  80
lys thr cys val ala asp glu ser ala glu asn cys asp lys ser leu his thr leu phe gly asp lys leu cys thr val ala thr leu
AAA ACA TGT GTT GCT GAT GAG TCA GCT GAA AAT TGT GAC AAA TCA CTT CAT ACC CTT TTT GGA GAC AAA TTA TGC ACA GTT GCA ACT CTT (350)

81                                90  91                          100 101                                 110
arg glu thr tyr gly glu met ala asp cys cys ala lys gln glu pro gly arg asn glu cys phe leu gln his lys asp asp asn pro
CGT GAA ACC TAT GGT GAA ATG GCT GAC TGC TGT GCA AAA CAA GAA CCT GGG AGA AAT GAA TGC TTC TTG CAA CAC AAA GAT GAC AAC CCA (440)

111                               120         124                 130                                     140
asn leu pro arg leu val arg pro glu val asp val met cys thr ala phe his asp asn glu glu thr phe leu lys lys tyr leu tyr
AAC CTC CCC CGA TTG GTG AGA CCA GAG GTT GAT GTG ATG TGC ACT GCT TTT CAT GAC AAT GAA GAG ACA TTT TTG AAA AAA TAC TTA TAT (530)

141                               150                             160                             168 169 170
glu ile ala arg arg his pro tyr phe tyr ala pro glu leu leu phe phe ala lys arg tyr lys ala ala phe thr glu cys cys gln
GAA ATT GCC AGA AGA CAT CCT TAC TTT TAT GCC CCG GAA CTC CTT TTC TTT GCT AAA AGG TAT AAA GCT GCT TTT ACA GAA TGT TGC CAA (620)

171                   177         180                             190                                     200
ala ala asp lys ala ala cys leu leu pro lys leu asp glu leu arg asp glu gly lys ala ser ser ala lys gln arg leu lys cys
GCT GCT GAT AAA GCT GCC TGC CTG TTG CCA AAG CTC GAT GAA CTT CGG GAT GAA GGG AAG GCT TCG TCT GCC AAA CAG AGA CTC AAG TGT (710)

201                               210                             220                                     230
ala ser leu gln lys phe gly glu arg ala phe lys ala trp ala val ala arg leu ser gln arg phe pro lys ala glu phe ala glu
GCC AGT CTC CAA AAA TTT GGA GAA AGA GCT TTC AAA GCA TGG GCA GTA GCT CGC CTG AGC CAG AGA TTT CCC AAA GCT GAG TTT GCA GAA (800)

231                               240                 245 246     250     253                             260
val ser lys leu val thr asp leu thr lys val his thr glu cys cys his gly asp leu leu glu cys ala asp asp arg ala asp leu
GTT TCC AAG TTA GTG ACA GAT CTT ACC AAA GTC CAC ACG GAA TGC TGC CAT GGA GAT CTG CTT GAA TGT GCT GAT GAC AGG GCG GAC CTT (890)

261         265                   270                         278 279 280                             289 290
ala lys tyr ile cys glu asn gln asp ser ile ser ser lys leu lys glu cys cys glu lys pro leu leu glu lys ser his cys ile
GCC AAG TAT ATC TGT GAA AAT CAA GAT TCG ATC TCC AGT AAA CTG AAG GAA TGC TGT GAA AAA CCT CTG TTG GAA AAA TCC CAC TGC ATT (980)

291                               300                             310                 316                 320
ala glu val glu asn asp glu met pro ala asp leu pro ser leu ala ala asp phe val glu ser lys asp val cys lys asn tyr ala
GCC GAA GTG GAA AAT GAT GAG ATG CCT GCT GAC TTG CCT TCA TTA GCT GCT GAT TTT GTT GAA AGT AAG GAT GTT TGC AAA AAC TAT GCT(1070)

321                               330                             340                                     350
glu ala lys asp val phe leu gly met phe leu tyr glu tyr ala arg arg his pro asp tyr ser val val leu leu leu arg leu ala
GAG GCA AAG GAT GTC TTC TTG GGC ATG TTT TTG TAT GAA TAT GCA AGA AGG CAT CCT GAT TAC TCT GTC GTG CTG CTG CTG AGA CTT GCC(1160)

351                               360 361                     369 370                                     380
lys thr tyr glu thr thr leu glu lys cys cys ala ala ala asp pro his glu cys tyr ala lys val phe asp glu phe lys pro leu
AAG ACA TAT GAA ACC ACT CTA GAG AAG TGC TGT GCC GCT GCA GAT CCT CAT GAA TGC TAT GCC AAA GTG TTC GAT GAA TTT AAA CCT CTT(1250)

381                               390     392                     400                                     410
val glu glu pro gln asn leu ile lys gln asn cys glu leu phe glu gln leu gly glu tyr lys phe gln asn ala leu leu val arg
GTG GAA GAG CCT CAG AAT TTA ATC AAA CAA AAT TGT GAG CTT TTT GAG CAG CTT GGA GAG TAC AAA TTC CAG AAT GCG CTG TTA GTT CGT(1340)

411                               420                             430                         437 438     440
tyr thr lys lys val pro gln val ser thr pro thr leu val glu val ser arg asn leu gly lys val gly ser lys cys cys lys his
TAC ACC AAG AAA GTA CCC CAA GTG TCA ACT CCA ACT CTT GTA GAG GTC TCA AGA AAC CTA GGA AAA GTG GGC AGC AAA TGT TGT AAA CAT(1430)

441                       448     450                             460 461                                 470
pro glu ala lys arg met pro cys ala glu asp tyr leu ser val val leu asn gln leu cys val leu his glu lys thr pro val ser
CCT GAA GCA AAA AGA ATG CCC TGT GCA GAA GAC TAT CTA TCC GTG GTC CTG AAC CAG TTA TGT GTG TTG CAT GAG AAA ACG CCA GTA AGT(1520)

471             476 477         480                             490                                     500
asp arg val thr lys cys cys thr glu ser leu val asn arg arg pro cys phe ser ala leu glu val asp glu thr tyr val pro lys
GAC AGA GTC ACC AAA TGC TGC ACA GAA TCC TTG GTG AAC AGG CGA CCA TGC TTT TCA GCT CTG GAA GTC GAT GAA ACA TAC GTT CCC AAA(1610)

501                               510         514                 520                                     530
glu phe asn ala glu thr phe thr phe his ala asp ile cys thr leu ser glu lys glu arg gln ile lys lys gln thr ala leu val
GAG TTT AAT GCT GAA ACA TTC ACC TTC CAT GCA GAT ATA TGC ACA CTT TCT GAG AAG GAG AGA CAA ATC AAG AAA CAA ACT GCA CTT GTT(1700)

531                               540                             550                             558 559 560
glu leu val lys his lys pro lys ala thr lys glu gln leu lys ala val met asp asp phe ala ala phe val glu lys cys cys lys
GAG CTC GTG AAA CAC AAG CCC AAG GCA ACA AAA GAG CAA CTG AAA GCT GTT ATG GAT GAT TTC GCT GCT TTT GTA GAG AAG TGC TGC AAG(1790)

561                 567     570                             580
ala asp asp lys glu thr cys phe ala glu glu gly lys lys leu val ala ala ser gln ala ala leu gly leu ter          ter
GCT GAC GAT AAG GAG ACC TGC TTT GCC GAG GAG GGT AAA AAA CTT GTT GCT GCA AGT CAA GCT GCC TTA GGC TTA TAA CATCACATTTAAAAG(1883)

                  ter   ter
CATCTCAGCCTACCATGAGAATAAGAGAAAGAAAATGAAGATCAAAAGCTTATTCATCTGTTTTTCTTTTTCGTTGGTGTAAAGCCAACACCCTGTCTAAAAAACATAAATTTCTTTAA(2002)

TCATTTTGCCTCTTTTCTCTGTGCTTCAATTAATAAAAAATGGAAAGAATCTAA ...... 20 ...... AA (2078)
```

Fig. 1.1 The primary structure of human serum albumin: nucleotide sequence of HSA mRNA as determined from cloned cDNA (Dugaiczyk *et al.*, 1982).

residues, particularly the 17th and 18th cysteines, since the presence (Behrens: cys-lys-glu-pro-cys) or absence (Meloun) of intervening amino acids affects the nature of the loops created by disulphide bridges.

More recently, Dugaiczyk *et al.* (1982) used an alternative approach and evaluated the nucleotide sequence of HSA mRNA from recombinant cDNA clones. This sequence agrees well with that of Meloun but Behrens' report is less comparable.

However, some disagreements between the former two sequences still remain. These are detailed below with the sequence determined by Meloun in brackets:

94-gln(glu), 95-glu(gln), 97-gly(glu)
170-gln(glu), 464-his(glu), 465-glu(his)
501-glu(gln) and residues
364-370-ala-asp-pro-his-glu-cys-tyr are specified as
(his-asp-pro-tyr-glu-cys-ala).

In addition, the latest sequence has verified the absence of amino acids between cys$_{278}$ and cys$_{279}$, thereby establishing the near perfect homology in the triplet domain structure of the molecule. Fig. 1.1 shows the primary structure according to Dugaiczyk *et al.*

1.2.2 The Secondary Structure of Serum Albumin

The distribution of secondary structure in bovine serum albumin (BSA) is shown in Fig. 1.2.

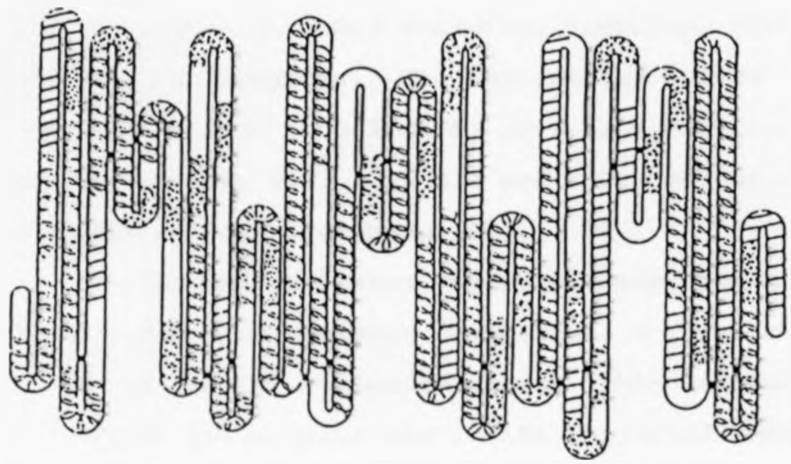

Fig. 1.2 Distribution of secondary structure in BSA (Peters and Reed, 1977).
Key: α-helix
β-pleated sheet
β-turns

Peters and Reed (1977) calculated the loci of β-turns, β-pleated sheet, α-helix and random coil using the revised sequence of BSA (Brown, 1977). The arrangement consists of long loops of α-helix modified with β-sheet and β-turn conformations, particularly in loops 1, 3, 6 and 7. The helix is predicted to extend to the short ends of some loops but model building demonstrates that it cannot continue for more than two residues beyond the cys-cys bond into these tight loops. Disturbance of the helices occurs at the apices of the loops, eight of which are formed into β-turns. Seven of the turns contain cystine residues and one includes the single thiol at cys_{34}.

An alternative structure proposed by Brown

(1977) and based on model building, comprises six uniform helical regions. The helices are joined at the end of each long loop by invariant proline residues (at 146, 222, 337, 414 and 530), and by disulphide bridges elsewhere (Fig. 1.3).

Further estimates employing model building and the amino acid sequence demonstrate a total of 46% α-helix and 16% β-pleated sheet. This compares well with 50-55% α-helix and 15-18% β-pleated sheet derived from optical measurements (Peters, 1975) although McLachlan and Walker (1977) suggest as much as 75% α-helix.

1.2.3 The Tertiary Structure of Serum Albumin

The regular occurrence of adjacent cys-cys sequences is paramount in the three dimensional organisation of the albumin molecule. Disulphide bridges formed between the 34 half-cystine residues form the molecule into three repeat units or domains, encompassing residues 1-190, 191-382 and 383-585 (Brown, 1975). Each domain consists of a large double loop, a short connecting segment, a small double loop, a long connecting segment, another large double loop and a connecting segment to the next repeat unit. Overall, the system involves a regular series of nine loops arranged to form the domains such that loops 1-3, 4-6 and 7-9 constitute domains I, II and III respectively.

There is no closure of the large loop

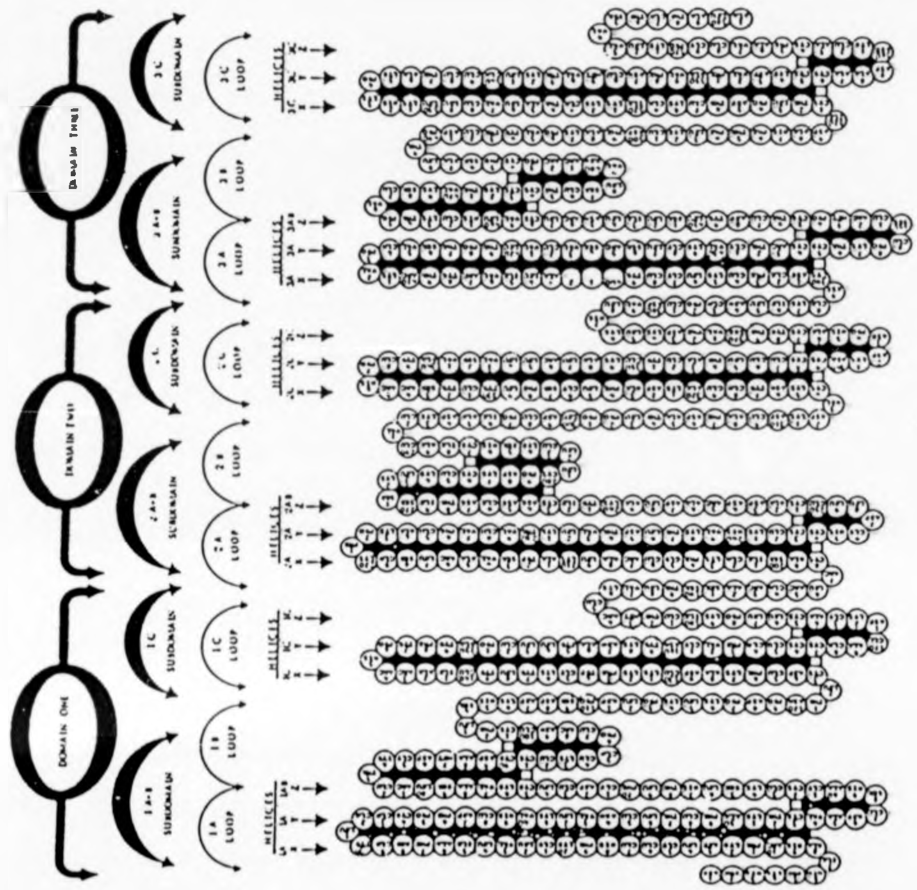

Fig. 1.3 The tertiary structure of bovine serum albumin (Brown, 1977).

between residues 8 and 54. Brown (1977a) believes that cysteine residues were once located at positions 8 and 54 but were lost during evolution.

Fig. 1.3 shows the triple domain structure and great internal homology of BSA according to Brown (1977). The absence of amino acids between cys_{278} and cys_{279} brings the structure of domain II closer to the structures of the other two domains. The locations of all 35 cysteine residues in both Meloun's and Dugaiczyk's sequence of HSA and Brown's sequence of BSA are exactly the same, consequently, the published tertiary arrangements are all identical.

There are considerable differences in composition among the domains which make the molecule asymmetrical. The frequency of tyrosine residues decreases from domain I to II to III. Tyrosine is particularly abundant in loops 3 and 6 whereas histidine is concentrated in loops 1 and 2. Each domain has a similar number of arginine and lysine residues but fewer aspartic acid and glutamic acid residues exist in domain III, hence, the C-terminal end is less negative than the N-terminal end of the molecule. Asymmetry of charges also occurs within individual loops. There is a tendency for basic residues to be positioned at the long ends of loops while acidic residues are situated at the short ends (Peters and Reed, 1977).

Each domain consists of two subdomains A-B and C containing a long loop and a hinge region. Each subdomain can be further subdivided into 3 helices X, Y and Z (Fig. 1.3).

The loop and link arrangement accounts for the flexibility and stability of albumin under conditions detrimental to other proteins: resistance to heat and to hydrogen bond disrupting agents (urea, guanidinium chloride), and the formation of isomeric forms as a result of expansion in acid or base. Local segments are maintained in near alignment by the disulphide bridges and isolated fragments containing disulphides retain most of their secondary structure and the ability to bind ligands whereas fragments without disulphide bonds do not (Reed *et al.*, 1975).

pH isomerisation (Zurawski and Foster, 1974) ranges from simple, reversible expansion to irreversible disulphide rearrangement. The normal 'N'-form predominates at pH 5-7. An expanded, faster migrating 'F'-form appears below pH 4, the expansion increasing below pH 3 to expose tyrosine and other hydrophobic residues. Near pH 8, particularly if calcium ions are present, a second type of expansion occurs to yield the 'B'-form in which hydrogen atoms become accessible for exchange. The mobility of the thiol group is increased and some of the helical conformation is lost. Above pH 8, a transformation to the slower moving 'A'-form prevails (Nikkel and Foster, 1971; Stroupe and Foster, 1973). This change involves disulphide bond rearrangement and becomes irreversible near pH 10.

Isolated and denatured fragments can regain their native disulphide pattern independently from the remainder of the molecule. Intact albumin does so more slowly (Teale and Benjamin, 1976).

1.3 CONFORMATION

The general shape of monomeric albumin can be represented by a prolate ellipse of dimensions 141 x 42 Å (Wright and Thompson, 1975). X-ray studies have further defined this structure as a linear arrangement of three spheres, 38-53-38 Å in diameter (Bloomfield, 1966) or more recently, fragmentation of native albumin has revealed a possible arrangement of four segments of unequal size with a small arm projecting from the N-terminal end of the first segment (Peters, 1970), Fig. 1.4.

The albumin dimer is a side-to-side aggregation of the monomer with an approximate overlap of 50% (Squire *et al.*, 1968).

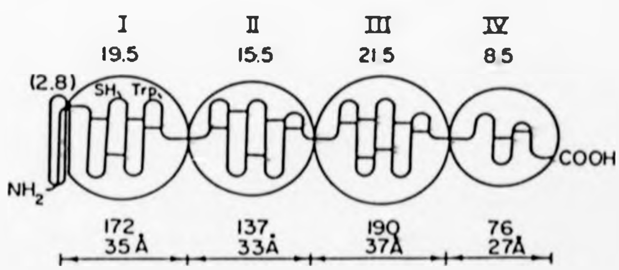

Fig. 1.4 The conformation of albumin (Peters, 1970).

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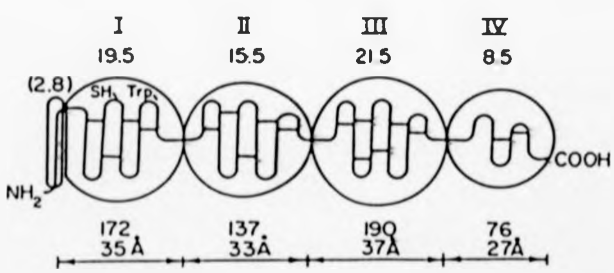


Fig. 1.4 The conformation of albumin (Peters, 1970).

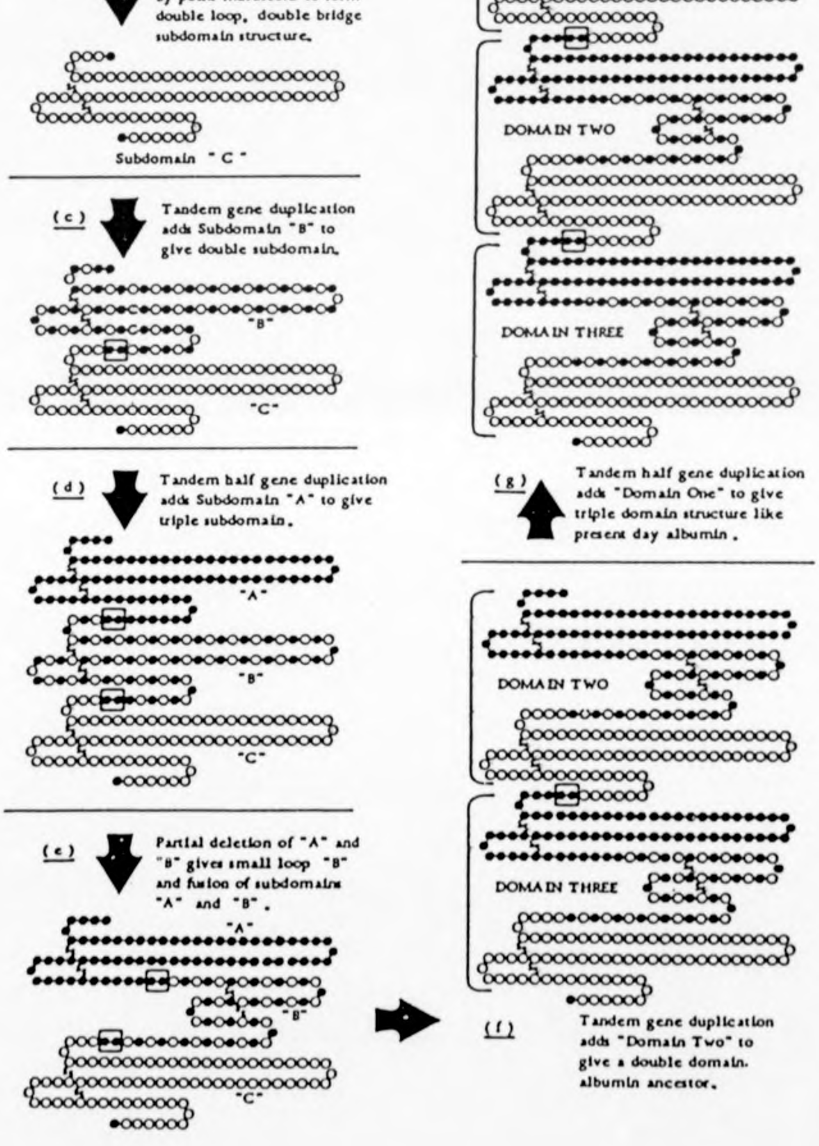


Fig. 1.5 Evolution of serum albumin (Brown, 1977).

1.4 EVOLUTION

The point during evolution at which albumin appeared is difficult to estimate. Proteins corresponding to human serum albumin have been observed inconsistently in the plasma of fish and amphibians (Cohen and Stickler, 1958; Engle *et al.*, 1958; Woods *et al.*, 1958), and Masat and Dessauer (1968) identified albumin in 15 species from the four major reptilian orders: turtles, crocodiles, lizards and snakes. Avian and mammalian albumins compare closely with HSA in solubility, size, charge and amino acid composition. Detailed interspecies comparisons using immunochemical methods have shown that albumin has evolved at a steady rate (Sarich and Wilson, 1967), through "neutral" (non-functional) amino acid changes (Peters, 1977).

Brown (1976) has proposed that the protein was built up to its present size by a nine-fold replication of a primordial subdomain structure comprising about 78 residues and 2 disulphide bridges. The greater similarity between domains I and II (domains I and II show 25% identity, II and III 21% identity and I and III only 18% identity) implies that the primitive gene corresponding to a single domain was duplicated to give the ancestral gene of domains II and III (about 156 residues). After sufficient time had elapsed to allow for divergence of these domains, a tandem half-gene duplication added domain I resulting in the triple domain structure reminiscent of present-day albumin.

Formation of the subdomains arose by triplication of a primordial subdomain gene corresponding approximately to the sequence 504-581, resulting in three large loops with long connecting segments (about 22 residues). Later, a deletion of about 40 residues near the middle of the sequence resulted in the large-small-large double loop pattern characteristic of the basic single domain (Brown, 1976a; 1977).

Brown (1977) proposed that the primordial subdomain probably arose from a gene fragment of a primitive globin about 1.1 billion years ago. However, statistical analysis performed on the sequence homology between the domains suggested rather, that the primordial double loop had the ability to dimerise and subsequent gene duplication allowed similar paired structures to form many times within a single, long chain, thereafter evolving its helical structure independently (McLachlan and Walker, 1977).

The approximate age of the triple domain structure is 700 million years. The evolutionary pathway of albumin as proposed by Brown (1977) is shown in Fig. 1.5.

1.5 PHYSICAL AND CHEMICAL PROPERTIES

Serum albumin is distinguishable from the majority of other plasma proteins by its smaller size, greater total charge, net charge at blood pH, greater symmetry, lower viscosity and higher solubility and stability (Cohn, 1941). It contains only 0.08%

carbohydrate, 0.02% trace metals and 0.8% fatty acids (calculated as oleic acid and equivalent to 1.8 moles/mole of albumin) (Goodman, 1957). Chen (1967) recognised from 1.8 to 2.5 moles per mole of protein in some preparations. Albumin is thus a simple protein consisting almost exclusively of amino acids.

The high content of glutamic acid, lysine and histidine is responsible for the high charge, polarity and ability to bind small molecules. The high proportion of cysteine provides 17 disulphide bridges per molecule. Comparatively low levels of glycine and tryptophan partially compensate for the surplus of these four amino acids.

Albumin is highly soluble in aqueous solutions at neutral pH (compared to the globulins) and more soluble than most other proteins at high salt concentrations. A unique feature is its solubility in mild organic solvents, e.g. 40% methanol at neutral pH.

At blood pH, the net charge on the molecule is -19 which renders albumin the most rapidly anodic migrating protein of the major plasma components. At this pH, 181 groups are ionised, 81 positively and 100 negatively. The ionisable groups comprise 102 carboxyls (pK 4.1), 15 imidazoles (pK 6.3), 1 α-amino group (pK 7.8), 56 ε-amino groups (pK 9.5), 17 phenolic residues (pK 10.2) and 23 guanidines (pK > 12) (Alberty, 1953).

The molar absorptivity of albumin is one of the lowest of the serum proteins at 5.3, due to there being only one tryptophan in the sequence. Spectral perturbation by large solvent molecules (sucrose, polyethylene glycol) shows that one third of the 17 tyrosine residues lie near the surface between pH 4.2 and 8 (Herskovits and Laskowski, 1962). One of the surface tyrosines and the single tryptophan are situated in an exposed location near the single sulphydryl group (Ohkubo, 1969). Acidification exposes 3-5 more residues from the hydrophobic interior as the molecule expands, although complete denaturation is required to expose all the aromatic amino acids.

1.6 HETEROGENEITY

The heterogeneity of commercial albumin preparations arises through the presence of protein contaminants, non-protein contaminants and factors inherent in the albumin molecule itself.

Traces of heavy metals and some fatty acids have proven difficult to remove and some samples have been found to contain citrate, pyruvate, lactate, aspartate and α-ketoglutarate ions (Janatova, 1974). Electrophoretic techniques can detect contamination by transferrin, α-globulins, genetic variants, bisalbumins induced by bound ligands, pH isomers and proalbumins. Other proteinaceous impurities include insulin, proteolytic enzymes, antihaemophilic factor (Barrow *et al.*, 1972) and bacterial endotoxin (Philip *et al.*, 1966). Artefacts may also arise through glycosylation of the ε-amino groups of lysine residues, in particular lys_{525} (Garlick and Mazer, 1983). Glycosylated albumin accounts for 6-15% of total serum albumin in healthy adults (Day *et al.*, 1979).

The distribution of free sulphydryl groups is invariably fractional, typically 0.6-0.7 mol/mole of albumin (Simpson and Saroff, 1958) as a result of non-mercaptalbumin formation. Non-mercaptalbumin arises *via* mixed disulphide bonding between the free sulphydryl group at cys_{34} and either cysteine or glutathione in the plasma (Andersson, 1966). Alternatively, the free sulphydryl group can exist in higher oxidation states such as the sulphonic acid (Noel and Hunter, 1972).

The care taken during isolation and storage of albumin strongly influences the final extent of heterogeneity in a preparation. Dimers and higher oligomers form spontaneously upon storage of plasma. New plasma contains only 5-8% dimers and other aggregates (Hartley *et al.*, 1962). Irreversible loss of reactive sulphydryl groups occurs with storage and freeze drying. This loss is not compensated for by molecular aggregation and is attributed to non-mercaptalbumin monomers.

Further intrinsic variations result from 'molecular aging' or modification of functional side groups without peptide bond breakage (Wong and Foster, 1969). Common reactions include deamidation of asparagine and glutamine (Robinson *et al.*, 1970), acetylation of amino groups by aspirin (Hawkins *et al.*, 1969) and oxidation of the sulphydryl group, all of which may occur during prolonged storage of plasma or as a consequence of rigorous isolation procedures.

1.7 LIGAND BINDING SITES

1.7.1 Introduction

Fig. 1.6 summarises the locations of binding sites for particular ligands. These sites either lie on the surface of the molecule or are accessible to the surface and can be categorised as follows:

(A) hydrophobic, non-covalent sites for

(i) primary long chain fatty acids

(ii) bilirubin and certain drugs

(iii) indole compounds and certain other drugs

(B) covalent attachment sites for organic ligands including the thiol group

(C) chelation sites for divalent metals.

A large majority of the binding sites have been characterised to a greater or lesser extent but for reasons of space, detailed discussion as to the mode and position of binding will be limited to those ligands relevant to this work.

1.7.2 The Binding of Fatty Acids

1.7.2.1 Introduction

The plasma free fatty acid (FFA) is composed primarily of long chain fatty acids (FA) (C_{16} and C_{18}) released from adipose tissue, the predominant species

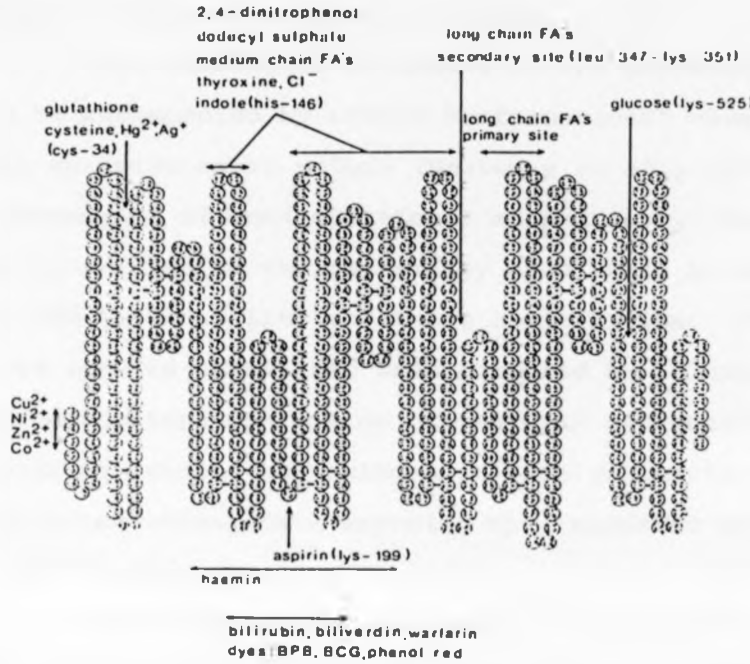


Fig. 1.6   The main ligand binding sites on HSA.

being oleate, stearate, linoleate, arachidonate and palmitate (Spector, 1971). Over two thirds are unsaturated and they are maintained in solution by non-covalent complexation of the anion monomers with albumin (Spector, 1975). Albumin can carry up to 2 moles of FFA without appreciably reducing its ability to bind other compounds.

Albumin functions to increase the availability of FFA to the cell membrane. It serves as a reservoir, effectively concentrating the FFA at the cell surface.

The formation of complexes permits maximum solubility and hence enhanced utilisation of FFA.

The binding of FA anions in the physiological range is accompanied by subtle conformational changes, namely an increase in volume (Soetewey *et al.*, 1972). The phenomenon of configurational adaptability (Karush, 1954) is related to the pliability of albumin binding sites and their ability to change conformation. The altered site is stabilised when occupied by an organic anion. Concomitant disruption of the weak intrahelical attractions permits the amino acid side chains to change orientation, thus exposing new, modified sites as the helices separate.

The Karush model accounts for cooperativity in binding and is more consistent with the mode of fatty acid binding than the Scatchard model which assumes a fixed number of pre-existing sites competing independently for the available ligand (Scatchard, 1949).

Such structural alterations are studied by analysing the perturbation of the fluorescence spectrum (Spector, 1968). The binding sites are mapped relative to the position of the single tryptophan residue. Alternatively, changes in the emission spectra of fluorescent probes: 8-anilino-1-naphthalenesulphonic acid (ANS) (Santos and Spector, 1974) and 5-(dimethylamino)-naphthalene-1-sulphonic acid (DNS) (Doody *et al.*, 1982) have been used to investigate fatty acid binding sites.

The presence of fatty acid anions stabilises albumin against the action of pepsin, trypsin, chymotrypsin, and other proteases (Kondo, 1962), against denaturation by heat, urea and guanidinium chloride (Boyer *et al.*, 1946; 1946a), and also facilitates realignment of the secondary structure during reoxidation of the completely reduced molecule (Andersson, 1969). Conversely, defatting increases the susceptibility of albumin to digestion by pepsin (Klapper and Cann, 1964).

1.7.2.2 Binding Models

Scatchard plots obtained for the binding of biologically important fatty acids show that a single albumin molecule possesses multiple sites for the accommodation of fatty acid anions. The plots are non-linear indicating that several types of site exist.

A number of binding models have been suggested (Klotz *et al.*, 1946; Goodman, 1958; Arvidsson *et al.*, 1971; Laiken and Némethy, 1971). These have been superceded by a generalised model (Spector and Fletcher, 1977) based upon stepwise addition of the ligand. No assumptions are made as to the pre-existence or independence of binding sites and configurational adaptibility, positive or negative co-operativity, site-site and ligand-ligand interactions are taken into account. The new scheme indicates that there are 6 high affinity and approximately 30 low affinity sites.

1.7.2.3 Structure of the Binding Sites

The sites themselves comprise two parts, a pocket lined with non-polar amino acid side groups and a cationic group located at the surface (Swaney and Klotz, 1970). The same authors demonstrated that one of the high affinity sites contains the sequence: lys-ala-trp-ala-val-ala-arg where the five non-polar side chains form the hydrophobic pocket and a cationic group is present at both ends.

Location is probably restricted to the hydrophobic clefts between adjacent globular domains. The single tryptophan residue is thought to reside in the cleft between the first and second domains (Brown, 1976a) and changes in the tryptophan emission spectrum induced by small quantities of FFA verify that one or more of the high affinity sites occur in this region (Santos and Spector, 1974).

The primary site for medium chain FFA's lies between residues 124-297 and 298-585. The secondary site is in the same general region (Lee and McMenemy, 1980).

1.7.2.4 Mechanism of Binding

Hydrophobic interactions between the fatty acid hydrocarbon chains and the non-polar amino acid side groups lining the binding site feature more prominently than electrostatic attraction or hydrogen bonding involving the carboxylate groups. The latter ionic interactions have been implicated in a minor capacity at the high affinity sites (Morrisett *et al.*, 1975).

The strongest hydrophobic interactions occur along the hydrocarbon chain, but the terminus, which penetrates furthest into the site, is not held firmly in place (Muller and Mead, 1973). Furthermore, the positioning of the hydrocarbon moiety is not consolidated by electrostatic attractions. At physiological concentrations only one mole of organic acid is contained within each binding site.

The association constants increase with increasing chain length: laurate < myristate < palmitate < stearate (Ashbrook *et al.*, 1975) and range from 2.4×10^6 to 2.6×10^8 M^{-1} for the primary sites and 3.5×10^3 to 3.9×10^5 M^{-1} for the weaker sites. Insertion of one *cis*-double bond increases the binding strength but a second double bond reduces affinity below that of the corresponding saturated fatty acid.

1.7.2.5 Palmitate

Palmitate has been used as a model for fatty acid binding and fragmentation studies have revealed a single, high affinity locus in or near loop 7 (Reed *et al.*, 1975). Further examination located the primary site to residues 377-503 (K_a 2×10^7 M^{-1}), the second site to residues 239-306 ($K_a = 8 \times 10^6$ M^{-1}) and the third site to the sequence 307-377 ($K_a = 2 \times 10^6$ M^{-1}).

1.7.2.6 Competition by other Ligands

Competition for binding has been observed with methyl orange (Cogin and Davis, 1951), ANS (Santos and Spector, 1972), bilirubin (Thiessen *et al.*, 1972), thyroxine (Tabachnick, 1964), and salicylates (Dawkins *et al.*, 1970) in the face of high concentrations of FFA. Drugs show little or no competition (Rudman *et al.*, 1971).

1.7.3 The Binding of Bilirubin

1.7.3.1 Introduction

Bilirubin features among the substances most tightly bound by albumin. Failure of albumin to bind this ligand in the plasma leads to hyperbilirubinaemia, the excess bilirubin being deposited in the extremities as jaundice takes effect. The condition is more serious in neonates in whom the immature blood-brain barrier lacks resistance to the passage of appreciable quantities of bilirubin, which subsequently becomes concentrated in the basal ganglia, causing kernicterus and possible lasting brain damage.

1.7.3.2 Location of the Binding Sites

The initial quantitative work concerning the interaction between bilirubin and albumin was carried out by Jacobsen (1969) who demonstrated that at least three moles of bilirubin were bound reversibly per mole of albumin at one high affinity site ($K_a = 7 \times 10^9\ M^{-1}$) and two equivalent low affinity sites ($K_a = 2 \times 10^6\ M^{-1}$). 1 mole of ligand bound at the primary site and 2 moles were detected within the weaker sites.

Fragmentation studies have enabled the binding sites to be located more exactly. Binding of bilirubin to large peptic (Geisow and Beaven, 1977) and small tryptic (Reed *et al.*, 1975) albumin fragments indicates that the primary site lies within the segment containing residues 186-238. This sequence approximately constitutes loop 4 of the tertiary conformation. However, photoactivated covalent binding of [^{3}H]-bilirubin to HSA located the primary site within the sequence 1-124 and a secondary site between residues 125-297 (Hutchinson and Mutopo, 1979). A second weak site was identified in the fragment 446-547 (Gitzelmann-Cumarasamy *et al.*, 1976).

In a physiological environment, the association constant for the primary site is in the order of 10^7-10^8 M^{-1} and that of the secondary sites in the order of 10^6 M^{-1} (Levine, 1977; Brodersen, 1979). However, Beaven *et al.* (1973) demonstrated that three sites exist at low ionic strength, namely one high affinity site (K_a = 1.4 to 2 x 10^7 M^{-1}) and two sites of lower affinity (K_a = 3.3 to 10 x 10^6 M^{-1} and 3.3 to 5 x 10^5 M^{-1}) whereas at high salt concentrations only two sites remain active, one strong site (K_a = 1.5 x 10^7 M^{-1}) and one weak site (K_a = 3.3 x 10^6 M^{-1}).

1.7.3.3 Structure of the Binding Sites

Chemical modification of certain amino acid residues of HSA have shown that arginine, tyrosine and histidine lie close to or are located within the high affinity site. Carboxyl, cysteinyl, and tryptophanyl groups are not involved (Jacobsen, 1972). Affinity labelling using reagents specific for the ε-amino groups of lysine suggest that two amino groups also form part of the primary site (Jacobsen, 1975), one of these being later localised to lysine$_{240}$ (Jacobsen, 1978).

Even if the tryptophan in loop 4 is not directly involved in binding, its environment is affected since attachment of the ligand quenches the intrinsic fluorescence of albumin. All mammalian albumins contain tryptophan near residue 214 (Feldhoff and Peters, 1976) although chicken albumin which has no tryptophan binds bilirubin equally strongly (Blauer *et al.*, 1977). The role of tryptophan in bilirubin binding as yet remains unclarified.

1.7.3.4 Mechanism of Binding

At physiological pH, bilirubin binds as the dianion (Hansen *et al.*, 1979; Jacoben and Brodersen, 1983). Lysine$_{240}$ is probably bound to one of the carboxylate groups by electrostatic attraction whereas a similar salt bridge to another lysine or the guanidinium group of an arginine may secure the second carboxylate function. Hydrophobic interactions have less influence on the stability of the bilirubin-albumin complex (Jacobsen, 1977).

1.7.3.5 Competition by other Ligands

Numerous anions compete with bilirubin and decrease the effective binding capacity of albumin. Among these are fatty acids, bile salts, haematin, acetamide and a number of drugs and azo dyes (Peters, 1970). Apart from long chain fatty acids, the affinity is generally lower than that for bilirubin.

Amongst the drugs, sulphonamides show widely differing displacement effects. Several analgesics, anticoagulants and antiinflammatory compounds including salicylates and food additives such as saccharin and antibacterial preservatives are also bilirubin displacers whereas benzodiazepines (Librium, Valium), most antibiotics, antihistamines, diuretics, general anaesthetics and hormones show little competition. Drugs carrying a net positive charge are not displacing (Brodersen, 1977).

Long chain (C_{16} and above) and short chain (C_{10} and below) fatty acids in excess of 4 moles/mole of albumin compete stongly for the primary bilirubin site. Conversely, the binding affinity is enhanced in the presence of medium chain fatty acids, e.g. laurate (C_{12}) and myristate (C_{14}) at a concentration of 2 molar. This positive co-operative effect is limited to a chain length of 12 to 14 carbon atoms (Brodersen, 1977a).

1.7.4 The Binding of Metals

1.7.4.1 Introduction

Albumin is a scavenger of heavy metal ions and prevents them from damaging the enzyme systems of the mitochondria. Metals interact with the free protein thiol groups in the organelle membrane (Verity and Gambell, 1968).

1.7.4.2 Copper

The binding of copper to albumin in the serum was first demonstrated by Bearn and Kunkel (1954). They showed that albumin was the immediate transporter of copper in the blood plasma following ingestion but later relinquished about 70% of it to the caeruloplasmin.

Copper binds at several sites along the albumin molecule. The primary site is located in the N-terminal tripeptide (NH_2-asp-ala-his) and involves four nitrogen ligands: the α-amino nitrogen, two intervening peptide bond nitrogens, and the imidazole nitrogen of the adjacent histidyl residue such that a square planar complex is formed. It has also been suggested that the side chain carboxyl group participates in the complexation of copper at this site (Sarkar, 1980) resulting in the penta-coordinated structure shown in Fig. 1.7.

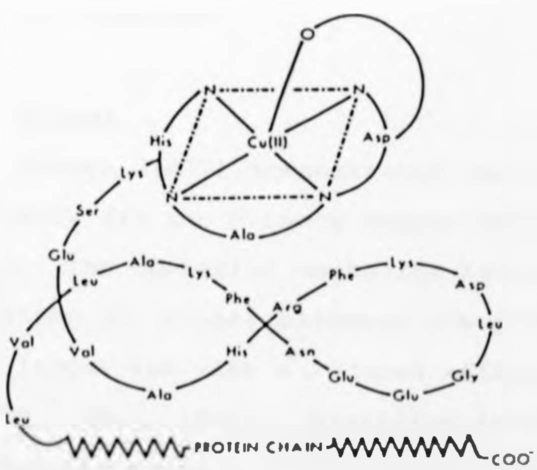


Fig. 1.7 The primary copper$^{(II)}$ transport site of HSA (Sarkar, 1980).

The identity of the first two amino acids is not restrictive since bovine and rat serum albumins bind copper equally well. Their tripeptide sequences are NH_2-asp-thr-his and NH_2-glu-ala-his respectively. However, the requirement of a histidine residue in the third position is illustrated by the relative failure of dog albumin (tripeptide sequence NH_2-glu-ala-tyr) to bind copper at the primary site (Appleton and Sarkar, 1971).

The association constant for the primary site is 10^8 M^{-1} (Breslow, 1964). The second equivalent of copper binds at the single sulphydryl group with a reduced affinity and can illicit the formation of albumin-S-Cu-S-albumin dimers analogous to the mercury dimer. The 4th to 9th binding sites have association constants of only 10^4 M^{-1} (Lal, 1959).

The major inhibitor of copper binding is the sulphide ion (Cartwright *et al.*, 1954). Cupric sulphide is insoluble and renders the copper unavailable for the formation of complexes.

1.7.4.3 Nickel

Peters (1977) demonstrated that nickel$^{(II)}$ can sterically fit the primary copper binding site of albumin. The chelation mechanism is similar to that operating for copper although the protein accommodates the larger ion with a reduced affinity, K_a = 7.5×10^3 M^{-1} (Rao, 1962). Histidine in the third position is also mandatory for nickel complexation.

1.7.4.4 Other Metals

The reaction of albumin with metal ions other than the first one or two ions of copper, nickel and mercury are more electrostatic in nature and of lower affinity. Silver$^{(I)}$ binds with high affinity but being monovalent, it cannot cause dimerisation.

Metal binding is minimal below the isoelectric point of albumin and increases with pH. The number of sites available for binding zinc$^{(II)}$ and cadmium$^{(II)}$ is small (Perkins, 1961), the principal groups involved being imidazoles (Gurd and Wilcox, 1956). Guthans and Morgan (1982) showed that 2-3 moles of $Zn^{(II)}$, $Ni^{(II)}$ and $Cd^{(II)}$ are bound per mole of albumin with association constants in the range 10^6 M^{-1} and relative affinities $Zn^{(II)} > Ni^{(II)} > Cd^{(II)}$. Lead$^{(II)}$, calcium$^{(II)}$ and magnesium$^{(II)}$ bind at a complex of carboxyl groups (Gurd and Murry, 1954; Irons and Perkins, 1962).

1.7.5 The Binding of Dyes

1.7.5.1 Binding Sites

Albumin binds a multitude of synthetic and exogenous anions including many organic dyes and pH indicator substances. In general, associations are weaker than those observed for bilirubin and fatty acids and the number of binding sites varies according to the dye.

Competitive binding studies indicate that several dyes, particularly the phenolsulphonephthalien group, e.g. phenol red, bind to a region common to the primary bilirubin binding site (Kragh-Hansen *et al.*, 1974). Phenol red and the tetrabromo-derivative bromophenol blue are bound with high affinity at 5 common sites, the association constants being approximately 100-1000-fold smaller for phenol red than for bromophenol blue.

Sulphobromophthalien interacts at a bilirubin binding site distinct from the site of interaction of indocyanine green, bromocresol green and rose bengal. The former shows a greater preference for the secondary bilirubin sites whereas the latter dyes associate more strongly with the primary site (Kamisaka *et al.*, 1974). Bromocresol green was observed to bind to albumin fragments (Reed *et al.*, 1975). Binding was proportional to fragment size but occurred to the greatest extent in the region of bilirubin binding.

2-(4'-Hydroxybenzeneazo)benzoic acid (HABA) binding is dependant upon albumin concentration (Bowmer and Lindup, 1980). Both the association constants and number of high affinity sites decrease with increasing protein concentration although the secondary sites remain largely unaffected. This anomaly could not be adequately explained by the presence of impurities, by aggregation or by competition with other ligands. Inhibition by chloride ions was also observed, however, none of these conditions influenced the uptake of bromocresol green or methyl orange.

The naphthalenesulphonic acids, Trypan blue, Evans blue and Congo red are tightly bound (Flanagan and Ainsworth, 1968) as are the azobenzoates such as methyl red (Burkhard *et al.*, 1961). The mode of m-methyl red interaction differs from that of o- and p-methyl red. The isomers do not compete among themselves for sites, suggesting non-identical binding loci. Trypan blue binds to a maximum of three sites but as saturation approaches, two primary sites are replaced by three weaker sites, possibly as a result of competition by the functional groups of the dye.

Like other ligands, dyes cause configurational changes in the albumin which stabilise it towards denaturation and proteolytic digestion (Markus *et al.*, 1967).

1.7.5.2 Competition by other Ligands

ANS, dodecylsulphate and dodecylsulphonate displace phenol red competitively from the high affinity sites. ANS competes at 4-5 sites. Bilirubin inhibits binding at equimolar concentrations. Palmitate and oleate show modest competition above a four-fold molar ratio and iopanoate shares a mutual high affinity site with bromophenol blue (Kragh-Hansen, 1981).

1.7.6 The Binding of Antigen

1.7.6.1 Introduction

Antigenic sites on albumin are specific binding loci, whereby the antibody is regarded as the macromolecule and the albumin site as the ligand. These sites have been located through fragmentation studies and immunologically active fragments have been isolated from different parts of the albumin molecule.

1.7.6.2 Location of Binding Sites

The amount of antigenic activity is directly related to the size of the fragments. Isolated and reduced domains are able to refold independently of the remainder of the polypeptide chain to reform the native antigenic structure, the third domain regaining its native conformation more rapidly than the first domain (Teale and Benjamin, 1976). In addition, the two halves of BSA can recombine to regenerate a complete antigen (Peters and Reed, 1977).

Peters *et al.* (1977) studied the antigenic behaviour of 14 fragments of BSA and predicted at least 6 antigenic determinants distributed fairly uniformly along the entire length of the molecule, with the site of greatest antibody affinity situated in the C-terminal region. Likewise, Bellon and Lapresle (1975) demonstrated the presence of two strong sites in loop 9 of HSA. By correlating available information Peters and Reed (1977) assigned the antigenic sites to residues 1-49, 50-184, 185-306, 307-385, 386-504 and 505-582.

1.7.6.3 Characterisation of the Binding Sites

The nature of the antigenic sites is unclear. Habeeb and Atassi (1976) proposed that native albumin carries identical, repeating sites based upon the loop- and link structure of albumin. However, Peters *et al.* (1977), although confirming the existence of multiple, homologous sites which react with the same populations of antibodies, found evidence for unique determinants which combine selectively with an exclusive antibody population, and further suggested that each half of albumin has its own characteristic sites which do not react with antibody to the other half.

Doyen *et al.* (1982) presented a compromise view. Using CNBr fragments of HSA, they observed that antibodies not only combined strongly with the fragments from which they were derived (homologous), but also showed a weak cross-reactivity with the other fragments (heterologous), the latter reaction resulting from the homology inherent in the albumin molecule.

The antigenic sites appear to be negatively charged. Neither intact tertiary structure, nor amino, phenolic or sulphydryl groups are essential but free carboxyl groups are a certain requirement (Sri Ram *et al.*, 1962). Antibodies may bind to homologous sites if the residues therein are similar in size and charge and the difference between homologous and identical sites may merely be a question of affinity.

1.7.7 Other Ligands

Kragh-Hansen (1981) proposed the existence of at least 6 binding regions on albumin. A general feature of albumin-ligand interactions is the presence of one or two high affinity sites accompanied by a variable number of weaker sites, depending on the ligand. Further, at high ligand to albumin ratios, several additional ligand molecules can associate weakly in a non-specific manner. Some ligands, e.g. dodecylsulphate and ANS bind with equal affinity at several sites, 8 for dodecylsulphate (Ray *et al.*, 1966) and 4 for ANS (Kolb and Weber, 1975).

Apart from the binding regions shown in Fig. 1.6, other regions as yet uncharacterised are of major importance in the transport of negatively charged and electrically neutral drugs such as warfarin, phenylbutazone, indomethacin and paracetamol. Positively charged drugs, quinine, procaine and the antimalarial compound pamaquine associate with comparable strength but at different locations.

Low concentrations of steroids bind tightly to the globulins but associate mainly with albumin when present at high levels. Progesterone and testosterone interact with secondary fatty acid binding sites and several steroids can bind to the primary bilirubin site.

1.8 ALLOALBUMINAEMIA

1.8.1 Introduction

Alloalbuminaemia may be defined as the presence of either two allomorphs - or one variant form of albumin in body fluids, the most frequent observations being made in the serum. On electrophoresis, the former heterozygous type appears as two bands showing varying degrees of separation, one corresponding to the common albumin A and the second migrating either ahead of (the so-called fast or anodic variants) or behind the normal component (the so-called slower, cathodic albumins, appearing between albumin A and α_1-globulin). Homozygote patterns are extremely rare and difficult to detect unless the normal allotype is run in parallel for comparison. Careful scrutiny and constancy in sample application is required for these.

The difference in electrophoretic mobility is brought about by a difference in net charge between the two forms.

Albumin variants fall into three categories:

(A) Congenital, hereditary alloalbuminaemia

(i) Structural alloalbuminaemia - arising from a small change within the amino acid sequence.

(ii) Dimers - monomeric variants with an increased tendency to dimerise.

(B) Acquired, transitory bisalbuminaemia - due to the binding of small organic ligands or as a consequence of enzyme action during pancreatic disease.

(C) Analbuminaemia

1.8.2 Congenital, Hereditary Alloalbuminaemia

1.8.2.1 Introduction

A true, hereditary alloalbumin should be distinguished from transient variants, dimers formed upon prolonged storage of serum, pH isomers ('F'-, 'A'- and 'B'-forms), electrophoretic bands split by protein-buffer interactions (Cann, 1966), prealbumin, α-fetoprotein and α_1-globulin which runs close to albumin on filter paper.

Numerous analyses concerning the pedigrees of families with alloalbuminaemic members have shown that the albumin phenotype is transmitted by two co-dominant alleles. The trait is not sex-linked. The propositus is usually heterozygous for the condition although a number of homozygotes are known. No incidences of heterozygotes for two different variants have as yet been found.

1.8.2.2 <u>Mechanism of Mutation</u>

Available structural information indicates that albumin variants arise through a single amino acid change at some position in the sequence.

Single point mutations result from a single base change within the gene. In transitional mutations, one purine-pyrimidine base pair is replaced by another. Transversional mutations occur when a purine-pyrimidine pair is substituted for a pyrimidine-purine pair. Both types of mutation can occur spontaneously and are relatively benign since only one amino acid is altered without affecting the remainder of the sequence. The defective protein is usually still functional.

Certain amino acid substitutions require more than one base change and are therefore less likely to occur, e.g. asp → gly is more likely than asp → met,

→ phe or → leu.

Only the following substitutions have been identified:

(i) Albumin B (Oliphant) (Winter *et al.*, 1972)
glutamic acid (GAA, GAG) → lysine (AAA, AAG)

(ii) Albumin Mexico-2 (Franklin *et al.*, 1980)
aspartic acid (GAU, GAC) → glycine (GGU, GGC, GGA, GGG)

(iii) Proalbumin Christchurch (Brennan and Carrell, 1978)
arginine (CGU, CGC, CGA, CGG, AGA, AGG) → glutamine (CAA, CAG)

(iv) Proalbumin Lille (Abdo *et al.*, 1981)
arginine → histidine (CAU, CAC)

Gentou and Plazonnet (1978) observed a substitution of 10 glutamic acid and/or glutamine residues by an equal number of lysine residues and of 5 alanines by prolines in an un-named slow variant. They attributed the change to an inversion of a cistron segment GAA (glutamic acid) → AAG (lysine) and GCC (alanine) → CCG (proline).

1.8.2.3 <u>Linkage to other Loci</u>

Pedigree data have been investigated for possible linkages between the albumin locus and other genetic systems. Weitkamp *et al.* (1966); (1968) and Kaarsalo *et al.* (1967) recognised an association between the albumin and the Gc (group specific component (Hirschfeld, 1962)) loci. The probability of linkage with hereditary methaemoglobinaemia (Scott and Wright, 1969), haptoglobin and transferrin (Kaarsalo *et al.*, 1967) loci is small but Keuppers *et al.* (1969) tentatively suggested a relationship with the α-chain of haptoglobin. Apart from these connections, albumin variants appear to arise independently of other genetic variations in proteins.

1.8.2.4 Alloalbuminaemia in Man and other Animals

1.8.2.4.1 Presence in Animals Controlled genetic studies in animals have confirmed that albumin alleles are autosomal and co-dominant. Alloalbuminaemia occurs in cattle (Ashton, 1964), sheep (Tucker, 1968), pigs (Kristjansson, 1966), horses (Stormont and Suzuki, 1963), turkeys (Quinteros *et al.*, 1964), chickens (McIndoe, 1962), quail (Haley, 1965) and doves (Miller, 1967). Hybrid crosses between domestic fowl show that three phenotypes are possible, one heterozygous and two homozygous forms.

In horses and sheep, three co-dominant alleles A, B and C can be demonstrated. Appropriate crosses can yield three heterozygous (AB, AC, BC) or three homozygous (AA, BB, CC) progeny.

In pigs the O allele controls no detectable albumin synthesis so that animals with the OO genotype are analbuminaemic and heterozygous AO and BO animals had albumin levels decreased to about half those with the AA, AB or BB genotypes.

A high degree of albumin polymorphism is detectable in toads (Guttman and Wilson, 1973).

1.8.2.4.2 Presence in Man The first case of allo-albuminaemia was reported by Scheurlen (1955) in a Swiss-German diabetic patient. The appearance of two albumins in the serum coincided with the onset of a diabetic coma, the electrophoretic pattern returning to normal once the illness had stabilised. Two years later, Knedel (1957) and Nennstiel and Becht (1967) simultaneously recognised the hereditary nature of the condition, through studies of slow and fast variants of German origin. Following these observations, Wuhrmann (1959) reinvestigated Scheurlen's patient. Her double albumin pattern had become permanent and was present in other members of her family. These findings substantiated the previous evidence for a hereditary phenomenon. The failure to detect the albumin variant during periods of stability was probably due to the lack of resolution inherent in filter paper electrophoresis.

Earle *et al.* (1959) described a slow variant, the original albumin B, in a Norwegian-American family. The anomaly was present in 25 out of 43 members of the family. From several immunological studies, they concluded that the alloalbumin B was antigenically indistinguishable from the normal form. The new protein described by Fraser *et al.* (1959) was probably the first dimeric variant to be found.

The genetic hypothesis gained support as additional cases of uncommon albumin bands were reported. Apart from those of German origin, slow-moving variants (albumins B) were found in Europeans from several countries including

France (Sandor *et al.*, 1965), Italy (Sarcione and Aungst, 1962), the U.K. (Franglen *et al.*, 1960; Cooke *et al.*, 1961), Sweden, Norway (Efremov and Braend, 1964) and Denmark (Drachmann *et al.*, 1965) and in the United States (Robbins *et al.*, 1963).

Two fast albumins were discovered in European families, albumin Gent (Wieme, 1960) in a Belgian family and albumin Reading in a Welsh/English family (Tárnoky and Lestas, 1964).

The first non-European variant was discovered by Melartin and Blumberg (1966). This fast alloalbumin was found among American Indians and was termed albumin Naskapi. It differed from those previously reported both in electrophoretic mobility and the relatively high frequency at which it occurred. Albumin Naskapi was also the first observation of an individual homozygous for a variant albumin.

By 1967, a sizeable literature concerning albumin variation had accumulated and was reviewed by Melartin (1967). Since then, the number of incidences reported has increased greatly due to the application of straightforward electrophoretic screening techniques to sera in many parts of the world, and the subject has been reviewed more recently by Tárnoky (1980).

New variants have been found among the Japanese (Fukunaga *et al.*, 1973; Nishimukai *et al.*, 1982), Malaysians (Lie-Injo *et al.*, 1971; Welch and Lie-Injo, 1972), Indonesians (Lie-Injo *et al.*, 1974), Indo-Dravidians (Tárnoky and Dowding, 1969; Bradwell *et al.*, 1975;

Frohlich *et al.*, 1978) and South Americans (Weitkamp and Chagnon, 1968; Arends *et al.*, 1970; Salzano *et al.*, 1974).

The distribution of albumin variants appears to be world wide. However, only two cases of alloalbuminaemia in individuals of African ancestry have been observed (Cohen, 1965; Weitkamp *et al.*, 1969). This can probably be explained by the paucity of observations in this region.

1.8.2.5 Nomenclature

The terms 'split'- or 'double'-albumin and 'iso'-, 'para'- or 'bisalbuminaemia' have all been used in early attempts to describe albumin variants. These have been superseded by the term 'alloalbumin' for forms other than the normal allotype.

The first notations distinguished only two albumins, the normal albumin A or A_1 and the variant type called fast, slow, A_2 or B, later expanded to include faster and very fast in attempts to classify the increasing confusion of variants. The accepted notation is that proposed by Melartin (1967) whereby normal albumin is designated albumin A (gene symbol Al^A) and variants are named after their ethnic, geographical or laboratory origin with the exception of albumin B which is retained exclusively for the Norwegian-American slow albumin described by Earle *et al.* (1959).

The normal genotype becomes Al^A/Al^A. Similarly, the genotypes for heterozygotes, e.g. albumin Redhill are Al^{A}/Al^{Rd} and for homozygotes, e.g. albumin Naskapi, Al^{Na}/Al^{Na}. Italian cases are described by laboratory location followed by the family's place of origin; the letters are taken from car number plates.

1.8.2.6 Classification of Variants

The majority of cases of alloalbuminaemia are discovered in hospital laboratories during routine electrophoresis of sera at pH 8.6. Others are especially looked for in an experimental capacity by electrophoretic screening of selected populations or racial groups. Several methods for the characterisation of variants have been devised.

1.8.2.7 Classification of Variants by Electrophoresis

Electrophoretic analysis is the most commonly used criterion for establishing the nature of variants.

Variants migrating in close proximity to Albumin A may show up merely as widened bands and serum dilution may be necessary. Where the alloalbumin has arisen *via* an amino acid substitution involving no net change in charge, its mobility remains that of albumin A. Such electrophoretically silent variants require alternative procedures, such as peptide mapping, for their detection.

There are two standard procedures for classification by electrophoresis: the starch gel system of Weitkamp *et al.* (1973) at three pH levels, and the Royal Berkshire Hospital (RBH) test schedule (Tárnoky, 1980) which involves dye binding and electrophoresis on six media, all at alkaline pH.

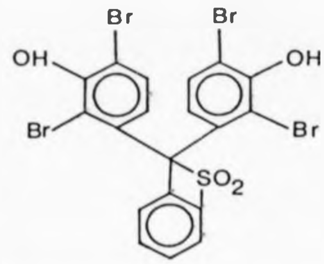
1.8.2.7.1 Starch Gel Electrophoresis (Weitkamp *et al.*, 1973) Variants are compared in three vertical starch gel systems: acetate-EDTA at pH 5.0, *tris*-lithium-succinate-citrate at pH 6.0 and *tris*-EDTA-borate at pH 6.9. Of these, the pH 5.0 separation is superior for distinguishing fast variants whereas the pH 6.0 and 6.9 systems are valuable for slow types.

Relative mobilities differ appreciably over this pH range leading in some cases to actual reversal in mobility of variants relative to each other in different buffer systems (Weitkamp and Chagnon, 1968; Weitkamp *et al.*, 1969). Alterations in electrophoretic conditions, e.g. extended running time, has a marked effect on the differential mobility of variants, possibly due to a changing environment within a discontinuous buffer system.

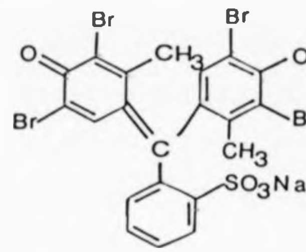
The use of several buffer pH's has increased the number of distinguishable types: no more than 12-15 variants are recognisable on any one system, whereas the combination of all three systems has realised some 28 allotypes.

1.8.2.7.2 The RBH Test Schedule (Tárnoky, 1980) Variant sera are subjected to electrophoresis on the following six media:

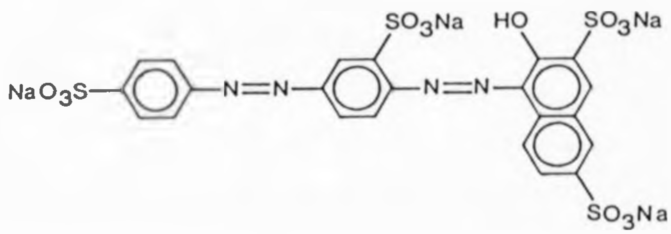
(i) Whatman 3 MM filter paper in barbitone buffer (0.05 M, pH 8.6).



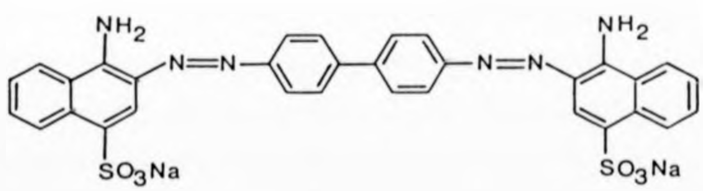
bromophenol blue



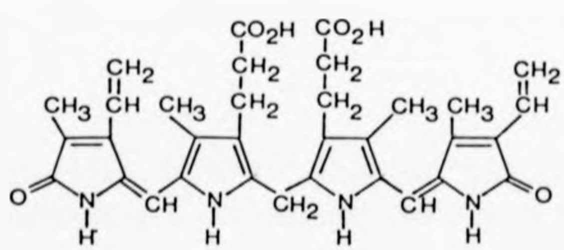
bromocresol green



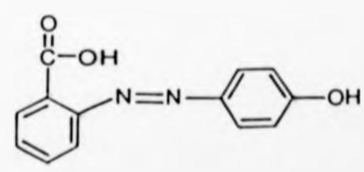
Ponceau S



Congo Red



bilirubin



2-(4′-Hydroxybenzeneazo)benzoic acid

Fig. 1.8 Classification of variants by dye binding: structures of the RBH test dyes.

(ii) Cellulose acetate (Shandon Celagram cellulose acetate strips) in barbitone Oxoid buffer (0.1 M, pH 8.6).

(iii) Helena Titan III Zip Zone cellulose acetate plates in barbitone buffer (0.05 M, pH 8.8)

(iv) Agar (1%) in barbitone buffer (0.05 M, pH 8.6) (Izquierdo *et al.*, 1971).

(v) Agarose (1%) containing sucrose (5%), EDTA (0.035%) and barbital (0.065 M, pH 8.6) in barbitone buffer (0.1 M, pH 8.6), using the Corning ACI electrophoresis system.

(vi) Disc-polyacrylamide (7%) in a *tris*-glycine discontinuous buffer system (pH 8.3 → 8.9 → 8.3) (Tárnoky and Dowding, 1967).

Relative mobility is expressed on a 0-100 scale, where 0 is the cathodic trailing edge of the serum pattern and 100 is the leading edge of albumin A (Tárnoky *et al.*, 1970).

1.8.2.8 Dye Binding

Qualitative tests based on transport function are carried out by incubating variant sera with a panel of six ligands: bromophenol blue, bromocresol green, Ponceau S, Congo red, HABA and bilirubin prior to electrophoresis on six media. The resultant dye uptake is a combination of interactions between the dye, protein and support medium and is estimated visually. All dyes are added to an excess of albumin except for HABA.

1.8.2.9 Critique of Ligand Binding Methods

Variation between results depends upon the uniform availability of free binding sites on the albumin molecule. Binding tests use non-defatted albumin so that pre-existing ligands such as drugs, fatty acids and bilirubin may compete for or already occupy the dye binding sites to a greater or lesser extent.

However, the tertiary integrity of the undefatted structure outweighs the advantage of a more standardised form, depleted of stabilising ions and with possible deformed binding sites. Preliminary defatting followed by reconstitution to an agreed fatty acid content would ensure comparability in testing. The IFCC criterion for standardisation of albumin preparations is 1-2 moles of fatty acid per mole of albumin and a polymer content below 1% (Tárnoky, 1981).

Dye binding is reversible and as electrophoresis proceeds, the ligand becomes detached and migrates more rapidly than albumin to the anode. Dye detached from the slow component would be temporarily rebound by the fast band so that this band would seem to bind more ligand. This can be remedied by using a standard running length and field strength and specified media.

The differential binding of dyes to alloalbumins is valuable for establishing differences, but it cannot prove identities.

1.8.2.10 Additional Methods of Classification

1.8.2.10.1 Ligand Binding

(i) Thyroxine

[^{131}I]-Thyroxine uptake by variants is quantified either by scintillation counting, autoradiography or scanning (Sarcione and Aungst, 1962; Yamamoto *et al.*, 1977). Lalloz *et al.* (1983) reported a silent variant with a 20-fold affinity for thyroxine over albumin A. However, interpretation of results is complicated by albumin acting only as an overflow depot for this hormone.

(ii) Radioactive Ligands (Cavalli-Sforza *et al.*, 1977)

Plasma proteins were screened using a panel of 63 physiologically important radioactive ligands including vitamins, hormones, metal ions, drugs, amino acids, purines, pyrimidines, fatty acids and carbohydrates. Comparative binding ability was assessed by PAGE-autoradiography.

1.8.2.10.2 Tests of Stability

(i) Low Temperature Storage

Some alloalbumins gradually merge into albumin A after prolonged storage in the cold. This phenomenon is possibly a return to an electrophoretically silent variant. Although the amino acid substitution does not involve a change in net charge, it may promote other changes which cause an alteration in mobility such as the binding of ligands only to the variant. These ligands become detached during storage so that the variant reverts to its original mobility. Two such cases are albumin Paris (Lie-Injo *et al.*, 1971) and the equivalent albumin Gombak (Weitkamp *et al.*, 1973).

(ii) Freezing and Thawing

Sera are subjected to two-, ten-, and thirty-five-fold freezing (-20^{o}C) and thawing (37^{o}C) followed by electrophoresis against control sera. This procedure removes lipid from albumin and the instability of albumin Luarca (Izquierdo *et al.*, 1971) to this treatment was manifested by a decrease in the relative proportion of the allotype from 44% to 35%.

(iii) Thermal Stability

This procedure identifies dimeric variants. Sera are heated at 56^{o} for 30 minutes and 2 hours. Under these conditions, dimers are dissociated and the mobility of the allotype reverts to that of albumin A. The dimeric character of albumin Warao was uncovered in this manner (Arends *et al.*, 1969).

1.8.2.10.3 Molecular Weight

The monomeric or dimeric nature of variants is reliably determined by gradient-PAGE.

1.8.2.10.4 Proportion of Normal:Variant Albumins

Most variants constitute just under 50% of the total serum albumin. Table 1.1 denotes some exceptions.

Table 1.1

Variant	Normal	: Variant	Reference
Cuneo/Belluno	79%	21%	Vacca *et al.* (1974)
Genova/Catania	90%	10%	Porta *et al.* (1974)
RIH	64%	36%	Chapman *et al.* (1978)
Stirling	59%-66%	41%-33%	Curnow *et al.* (1978)
Vancouver	35%	65%	Frohlich *et al.* (1978)

The relative proportions of dimeric variants are usually low, e.g. albumin Warao comprises only 30% of the total serum albumin (Arends *et al.*, 1969).

1.8.2.10.5 Isoelectric Points

The determination of isoelectric points is potentially useful in the classification of variants but this parameter has rarely been determined. One objection is that artefacts may arise through the binding of ampholytes to native albumin (Foster, 1977; Wallevik, 1973) and even normal albumin shows multiple bands (Kaplan and Foster, 1971; Spencer and King, 1971) and has several published isoelectric points: pI 4.8 (Evenson and Deutsch, 1978), pI 4.7-5.3 (Valmet, 1969), pI 5.2 (Carlsson and Perlmann, 1969) and pI 4.78 and

4.84 (Giuliani *et al.*, 1978) for non-defatted HSA. Successful application of isoelectric focusing would require standardisation of experimental conditions.

Sudaka *et al.* (1976) used isoelectric focusing in the pH range 4-6 to measure the isoelectric points of albumins Pollibauer (pI 5.84) and Gainesville (pI 5.65).

Bradley and Hornbeck (1974) used pH-gradient elution on the strongly anionic ion-exchanger QAE-Sephadex to determine the isoelectric points of several fast and slow variants. However, reproducible pH gradients are difficult to achieve and some variants are sufficiently similar to albumin A as to prevent separation.

1.8.2.10.6 Immunological Character

The antigenic behaviour of albumin variants has been tested by reaction against anti-albumin A and by reaction of albumin A against anti-albumin variant. These tests have indicated that alloalbumins are antigenically identical to albumin A.

This observed similarity arises from several features. Albumin is believed to possess similar repeating antigenic sites (Section 1.7.6) so that a single amino acid substitution would be unlikely to alter the overall immunological character. The site of mutation may not be involved with the antigenic nature of the molecule, moreover, binding of the antibody to only a few sites could be sufficient for precipitation. Also, the techniques employed may not be sufficiently sensitive to detect minute differences in the sequence and the heterogeneous antibody preparations obtained by immunisation are relatively undiscerning, thereby allowing sufficient reactivity to form a precipitate.

There are however, two exceptions. Robbins *et al.* (1963) reported no distinction between the slow and normal albumin components in undiluted serum but two separate precipitin arcs were formed after a 1:30 dilution. This was attributed to a secondary precipitation with excess antibody (Payne and Dickinson, 1967), bearing in mind the possible existence of a unique antigenic determinant. Margni *et al.* (1970) found albumin A to be antigenically deficient relative to a slow alloalbumin.

A far more definitive method would be the use of monoclonal antibodies. Micheel *et al.* (1982) used ^{125}I-labelled and fluoresceinisothiocyanate (FITC)-labelled normal HSA to measure the antibody activity of anti-HSA hybridoma antibodies. These monoclonal antibodies detected antigenic determinants containing tyrosine or lysine as an essential part.

1.8.2.10.7 Structural Studies

The ultimate means by which variants can be distinguished is by amino acid sequencing. So far, the primary structure of only a few variants has been investigated: albumin B (Winter *et al.*, 1972), albumin Mexico-2 (Franklin *et al.*, 1980), albumin Naskapi (Franklin *et al.*, 1980a), proalbumin Christchurch (Brennan and Carrell, 1978), proalbumin Lille (Abdo *et al.*, 1981), the un-named variant of Gentou and Plazonnet (1978) and the partial structures of albumin Gainesville (Lapresle, 1977) and albumin Adana (Franklin *et al.*, 1980a).

1.8.2.11 <u>Population Distribution of Variants</u>

At present, it is difficult to determine the exact number of variants. Out of about 80 variants named, approximately 28 have been distinguished from each other (Weitkamp, 1973). This discrepancy is due to the use of several names to apply to a smaller number of variants. The table compiled by Schell and Blumberg (1977) includes variants which were not named by their discoverers, variants which may be rediscoveries of previously identified variants and variants which appear in comparative studies but which have not been described in the literature individually. The result of these practises is a proliferation of variant names, many of which are not commonly used or recognised.

Variants may be either rare or polymorphic depending on whether they occur with a frequency of 1% or more. These two classes may be subdivided on the basis of their distribution.

There are three classes of rare variants:

(i) unique or private variants which have been seen only in the family in which they were discovered;

(ii) restricted rare variants which occur in a small number of unrelated individuals of

Table 1.2 Population distribution of some alloalbumins (adapted from Weitkamp et al. (1973) and Schell and Blumberg (1977)

Race	Variant	Type	Distribution	Origin
Caucasian	Pollibauer	Slow	Unique	Austrian
	B (SO/CZ)	Slow	Rare, restricted	Various European
	Roma	Slow	-	Italian
	Gainesville	Slow	Rare, restricted	Irish
	Paris (Gombak)	Slow (silent)	Rare, unrestricted	French
	SO/BS	Slow	-	Italian
	Cartago	Slow	-	Spanish
	Reading (New Guinea, Syracuse)	Fast	Rare, unrestricted	Welsh
	Gent (Fast)	Fast	Rare, restricted	Belgian
	RIH	Slow	-	Norwegian
	'Dimer'	Slow	-	Welsh, Swedish
	Stirling	Slow	-	Scottish
	Yorkshire	Slow	-	English
	Amsterdam	Slow	-	German
Negroid	Cayemite	Slow	Unique	Haitian Negro
	Uinba	Slow	Unique	Native New Guinea
	New Guinea (Reading, Syracuse)	Fast	Rare, unrestricted	Native New Guinea
	'Dimer'	Slow	-	Negro (U.S.A.)
Mongoloid	Gombak (Paris)	Slow	Rare, unrestricted	Native Malaysian
	Kashmir (Afghanistan)	Slow	Rare, restricted	Indian
	Pushtoon	Slow	-	Indian
	Mexico	Slow	Polymorphic, dispersed	American Indian
	Medán	-	Rare, restricted	Chinese Malaysian
	Makú (Makiritare-2)	Fast	Rare, restricted	Native South American
	Makiritare-3	Fast	Unique	Native South American
	Makiritare-1 (Warao)	Dimer	Polymorphic, restricted	Native South American
	Naskapi (Mersin)	Fast	Polymorphic, dispersed	American Indian
	Yanomama	Dimer	Rare, restricted	Venezuela
	Yanomama-2	Slow	Polymorphic, restricted	Native South American
	Xavante	Slow	-	Native South American
	Jaffna	Slow (silent)	-	Sri Lanka
	Vancouver	Slow	-	Indian (Fiji)
	Birmingham	Slow	Unique	Indian (Punjab)
Unknown	Santa Ana	Slow	Unique	Mexican
	Belém I	Slow	Unique	Brazilian
	Belém II (Mexico)	Slow	Polymorphic, dispersed	Brazilian
	Belém III (Makú)	Fast	Rare, restricted	Brazilian

one ethnic group; and

(iii) unrestricted rare variants which occur in a small number of unrelated individuals of different ethnic groups.

Polymorphic variants may be restricted to one ethnic group and locality or dispersed among several groups and localities. Table 1.2 shows the population distribution of some alloalbumins, adapted from Weitkamp *et al.* (1973) and Schell and Blumberg (1977).

1.8.2.12 Albumin B

Albumin B is a broad classification of the rare variants most frequently found in Europeans. It has a wide distribution with an incidence of heterozygotes of 1:1000-1:10,000. This variant was designated 'very slow' by Weitkamp *et al.* (1969). Similarity between all the albumins B has not been proved but there is evidence to suggest that some of them may be the same variant.

By studying electrophoretic separation in the pH range 3.0-12.3, Earle *et al.* (1959) concluded that a carboxyl group had been substituted by tyrosine, cysteine or lysine. Later, Gitlin *et al.* (1961) demonstrated that between pH 4.00 and 8.43, albumin B possesses a net charge of +2 greater than albumin A, in accordance with a change from a single carboxyl group to a single lysine residue.

The strongest evidence for similarity between albumins B came from sequence analysis of albumins Oliphant and Ann Arbor, of German and Danish descent respectively.

Peptide mapping revealed a novel peptide having the sequence ala-lys-glu-gly-lys-lys-leu instead of the usual ala-glu-glu-gly-lys-lys-leu. Subsequent CNBr fragmentation localised the anomaly to the C-terminal end of the molecule. The point mutation corresponds to a substitution of a lysine residue for glutamic acid$_{570}$. These findings are analogous to those concerning the earlier B albumin. Furthermore, albumins Ann Arbor and Oliphant were proved to be identical.

Several B-type albumins have been found in France, including albumin Paris (Fine *et al.*, 1976). Albumins Paris and Gainesville have been subtyped B" and B' (Ott, 1974) in a similar manner to a group of non-identical B albumins reported around Stuttgart amongst which, a homozygote, albumin B Schönaich was discovered (Ott *et al.*, 1973).

The C-terminal CNBr fragment of albumin B migrates faster than the corresponding fragments of albumins A and Mexico-2 at pH 3.0, consistent with the glu → lys substitution (Franklin *et al.*, 1980).

1.8.2.13 Albumin Mexico

Albumin Mexico is a polymorphic variant occurring in several widely separated geographical areas. It was initially reported in several non-related sera in Mexico (Melartin *et al.*, 1967) but has since been discovered in American Indians from Middle America and as far south as Guatemala. A consideration of the distribution and high gene frequency is a useful indicator of past cultural influences, linguistic similarities, migrations and relationships among the native American populations. At pH 8.6, the variant migrates midway between albumins A and B.

Using a new screening procedure involving the analysis of CNBr peptides in an acid-urea-non-ionic detergent gel system, Franklin *et al.* (1980) have resolved two species of the variant, normally indistinguishable by conventional electrophoresis. These have been named albumins Mexico-1 and Mexico-2, but because all surveys have so far employed conventional methods, the proportion of each variant in a population is impossible to calculate.

Albumin Mexico-2 arises through a substitution of aspartic acid$_{550}$ by a glycine residue. The resultant loss of one negative charge accounts for the slower mobility of the native protein at pH 8.6 and the faster mobility of the associated CNBr peptide at pH 3.0.

In contrast, no such structural information could be obtained for albumin Mexico-1 since all the CNBr peptides migrate identically to those of Albumin A in this system. This indicates that an acidic residue may be replaced by a neutral one or that the asp $\rightarrow$ gly change is present but is obscured by a second substitution.

The mutation lies in the region of the primary palmitate binding site. The change between the helix-indifferent aspartic acid and the helix-breaker glycine is sufficient to cause a gap in the helix of about four residues. This may influence the binding of long chain fatty acids. Haemoglobin and thyroxine bind normally (Melartin, 1967) but bromophenol blue and warfarin bind less readily (Wilding *et al.*, 1977). The clinical implication of decreased binding is an increase in pharmacologically active free ligand leading to an enhanced response and possible toxicity. However, all homozygotes for the trait appear to be healthy.

1.8.2.14 Albumins Naskapi, Mersin and Adana

Together with albumin Mexico, albumins Naskapi and Mersin are the only widely distributed variants occurring at polymorphic frequencies. Albumin Naskapi was discovered by Melartin and Blumberg (1966) among the Naskapi and Montagnais Indians of Quebec and is found predominantly in the Indian populations of North America, Alaska and Canada. It occasionally co-exists with albumin Mexico in the south-west. Where this occurs, the proportions of the two variants relative to albumin A and to each other differ between each Indian group.

Apart from very low frequencies observed in Eskimos of mixed ancestry (Melartin *et al.*, 1968), albumin Naskapi was thought to be exclusive to American Indians. However, a similar variant, albumin Mersin has recently been identified among Eti Turks in southeastern Turkey (Franklin *et al.*, 1980a). Albumin Mersin migrates identically to albumin Naskapi at pH 8.6 and may be the same variant. Both are fast alloalbumins.

The site of mutation in both albumins has been localised to residues 373-389. The relevant CNBr peptide (residues 330-446) migrates more slowly at pH 3.0 than its normal counterpart which suggests a change from a basic residue, although the exact substitution site has not been identified.

Albumin Naskapi binds haemoglobin and thyroxine normally but shows a diminished affinity for warfarin. Conversely, bromophenol blue binding is increased.

Albumin Adana, also found among the Eti Turks and migrating similarly to albumin B, differs from the latter and albumin A by a substitution between residues 447-548.

1.8.2.15 Proalbumins Christchurch and Lille

Human proalbumin differs from albumin A in that it possesses an additional N-terminal hexapeptide with the sequence arg-gly-val-phe-arg-arg. Proalbumin is the intracellular precursor of albumin, and has been well characterised (Russel and Geller, 1975; Judah and Quinn, 1976). The propeptide contains an excess of positively charged groups and terminates with an arginyl residue at the site of cleavage, therefore being cleavable by intracellular serine proteases with a specificity similar to that of trypsin. Proalbumin can, in fact, be converted to albumin *in vitro* by mild treatment with trypsin (Judah *et al.*, 1973; Quinn *et al.*, 1975; Brennan and Carrell, 1980). The presence of the basic propeptide also causes the 'pro'-form of albumin to be more positively charged than the active form, permitting separation of the two forms by electrophoresis.

In size, proalbumin is indistinguishable from albumin and no immunological differences are detectable upon double diffusion in agar against anti-albumin (Russel and Geller, 1973). The conversion of proalbumin into the active form occurs in the Golgi apparatus, probably by non-specific tryptic-like proteolysis.

However, the discovery of proalbumins Christchurch and Lille has provided additional evidence concerning the mode of cleavage of the propeptide. The C-terminal basic arginyl residue of the proalbumin Christchurch propeptide has undergone a point mutation to a neutral glutamine, whereas the penultimate residue in that of proalbumin Lille has changed from arginine to the basic histidine. Both proalbumins are readily cleaved *in vitro* by trypsin but remain uncleaved *in vivo*, which strongly suggests that cleavage occurs by specific proteolysis with paired arginine residues being a prerequisite. This fits the proposal that cleavage is effected by a specific protease, possibly Cathepsin B (Judah and Quinn, 1978).

Both proalbumins constitute approximately 50% of the total serum albumin. At pH 5.0, proalbumin Lille migrates more slowly than proalbumin Christchurch.

Proalbumin Christchurch has lost the high affinity copper$^{(II)}$ and nickel$^{(II)}$ binding site since the attachment of the propeptide has blocked the α-amino nitrogen essential for metal binding (Brennan and Carrell, 1980). It hasn't, however, altered the conformation of albumin since palmitate, bilirubin, ANS and bromocresol green are all bound normally (Reed *et al.*, 1980).

1.8.2.16 Albumin Gainesville

This slow, monomeric variant occurs exclusively in European populations (Weitkamp *et al.*, 1973a) and was originally discovered in an American family of Irish descent (Lau *et al.*, 1969). It is a rare variant, occurring with a frequency of less than 1 in 1000 and has been characterised by Weitkamp *et al.*,(1969a).

Fragmentation by CNBr has enabled a partial localisation of the mutation (Lapresle, 1977). The change lies within the first 122 residues from the N-terminal end.

1.8.3 Non-Hereditary, Transient Bisalbuminaemia

1.8.3.1 Introduction

True, acquired transitory bisalbuminaemia was initially discovered in an 11-year old boy with ascites by Gabl and Huber (1964). The condition is rare, with only 64 reported cases from 1948 to 1976. All cases show common features: the transient bisalbumin appears during an acute, probably viral infection and only persists for a short while, disappearing sometimes even before the illness has completely subsided.

Transients can be formed through the binding of ligands, acute illness or may constitute their own type. Simultaneous familial investigations rule out the possibility that the trait is hereditary. In inherited alloalbuminaemia, the two components are usually present in equal proportions whereas the proportion of a transient bis-albumin is markedly less than that of albumin A. Also, drug and enzyme-induced forms do not always show two distinct bands unlike inherited variants. Both albumins are usually immunologically similar.

Porta *et al.* (1980) reviewed 19 cases of transient bisalbuminaemia, including 57 bisalbumins induced by drugs, 5 probably due to pancreatic disease and 7 with an unidentifiable aetiology.

Dye binding has been employed by Porta *et al.* (1980) as a means of distinguishing induced bisalbuminaemia from hereditary alloalbuminaemia. Bisalbumins have less affinity for bromocresol green and bilirubin than normal albumin and alloalbumins. Enzymatic digestion also reduces the dye binding capacity.

1.8.3.2 Ligand Effects

The binding of some small ligands to albumin gives rise to two bands on electrophoresis, one due to free albumin, and a faster migrating band containing the albumin-ligand complex. Their proportions depend upon the quantity of ligand present.

The most noticeable binding effect is that of penicillin, discovered by Arvan *et al.* (1968) in patients receiving large intravenous doses of Penicillin G and related drugs. Patients developed a fast albumin component which disappeared upon cessation of therapy. *In vitro* experiments using normal sera, heterozygous alloalbuminaemic sera (albumins B and Naskapi), and purified albumin solutions showed that the phenomenon is related to the concentration of antibiotic in the serum. The binding of Penicillin G and Cephalothin induced the formation of four bands in the variant sera. The increase in mobility is due to the additional negative charge conferred upon the molecule by the carboxylic acid group of the drug.

The rapid form was not observed when Penicillin G was inactivated with penicillinase prior to binding. The consequent opening of the β-lactam ring renders the drug non-functional. However, penicillinase had no apparent effect on pre-existing penicillin-induced bisalbuminaemia, indicating that binding to albumin offers protection to the immobilised β-lactam ring.

Penicillin G

Penicilloic acid

Penicillin G-related drugs: Penicillin O, Methicillin, Nafcillin and Ampicillin were similarly effective but streptomycin and chloromycetin were less successful.

Gibaud *et al.* (1973) reported 12 cases of transient bisalbuminaemia induced by heavy doses of β-lactam antibiotics.

1.8.3.3 Transient Bisalbuminaemia Associated with Pancreatic Disorders

A limited number of cases concerning the presence of a fast albumin in relation to pancreatitis have been reported. Shashaty and Atamer (1972) described two cases where the fast components were immunologically albumins, their proportions being directly related to serum amylase levels, but remaining well below 50% of the total albumin, even during hyperamylasaemia.

Lamotte-Barillon *et al.* (1975) observed a case of a different nature. The patient was suffering from chronic pancreatitis and ascites and the fast band was present continually, irrespective of amylase concentration. Since the electrophoretic pattern could be reproduced by partial digestion of albumin with trypsin *in vitro*, it was concluded that the fast component was an enzymic breakdown product of albumin.

Rousseaux *et al.* (1976) discovered transient bisalbumins in the serum, ascitic fluid and pleural fluid of three patients. Structural analysis of the fast component revealed that the C-terminal end of the molecule differed from normal albumin and attributed this to limited enzymatic degradation by the proteolytic enzymes of the pancreas: chymotrypsin or elastase, followed by the action of carboxypeptidases A and B.

Gastard *et al.* (1976) demonstrated the clinical importance of finding a bisalbumin. Appearance in a patient with ascites suggests a pancreatic disorder, whereas a pancreatic fistula may be suspected should it be observed during chronic pancreatitis. In addition, possible overdosage may be prevented in patients with renal insufficiency undergoing intensive drug therapy (Bismuth *et al.*, 1976).

A rare combination of pancreatic disease and hereditary slow alloalbuminaemia gave rise to accidental trisalbuminaemia (Tárnoky, 1982). The shortened albumin A molecule formed a fast band while the degraded slow variant migrated inside the unchanged albumin A band.

1.8.4 Analbuminaemia

Analbuminaemia is the absence or near absence of albumin in an individual. Plasma albumin concentration may be less than 0.5 g/litre (typically 1 mg to 100 mg %). In man, the term seems to describe two separate abnormalities. Christen and Franglen (1972) liberated a large quantity of previously undetectable albumin from a macroglobulin complex, suggesting that one form of the disorder may be a hereditary masking of plasma albumin. Alternatively, the illness is generally associated with a deficiency in albumin synthesis.

The trait is rare and assumed to be an inheritable, autosomal recessive abnormality since patients are often the offspring of consanguineous marriages (Ott, 1974a). Only 18 unrelated cases have been reported. Detection is usually delayed into adolescence but incidence in babies has been observed (Cormode *et al.*, 1975; Goulle *et al.*, 1976).

The lack of albumin is partly balanced by an increase in the other serum proteins: globulins, haptoglobulins, α_1-antitrypsin, caeruloplasmin, α_2-macroglobulin, IgM, transferrin, β-lipoprotein and fibrinogen. Some of the transport functions are taken over by other proteins. Alternative fatty acid transport is efficient, less so for bilirubin.

The small quantity of albumin present has a prolonged half-life: 40-110 days compared to the normal turnover rate of 20 days. The survival time of transferrin is also extended. The catabolic rate for both proteins may or may not return to normal after restoration of albumin levels by infusion (Gitlin and Gitlin, 1975) which in itself suggests two forms of the defect, possibly also a defect in albumin catabolism. However, Waldmann (1977), is critical of this notion since the studies were carried out at different times after infusion.

Despite the absence of a functionally important protein, sufferers complain of only mild symptoms: occasional fatique, fainting, some oedema and anaemia. Renal function is affected, low blood pressure and low capillary pressure are balanced by a low extravascular osmotic pressure. Circulating unbound bilirubin is a potential cause of jaundice and skin changes (11% of total body albumin is contained within the skin) such as eczema and dermatitis are occasionally presented.

Analbuminaemia is generally detected by immunological techniques and its existence, apart from being clinically important, leads to some speculation about the necessity and normal functions of albumin.

1.8.5 Effects of Variant Albumins

Few diseases can be linked with certainty to alloalbuminaemia. The main cause for concern lies with the altered binding properties of the variant albumin for drugs and bilirubin. Dosage calculations based only on the performance of normal albumin may lead to overdosage or undertreatment. The former is especially applicable to analbuminaemia. Reduced bilirubin binding may increase the risk of kernicterus in infants.

The reasons for variant polymorphism in American Indians as opposed to Europeans and other non-Indian populations is unclear. Blumberg (1969) speculated on the advantages of possible altered binding properties, particularly in relation to drugs. The retention of the Al^{Na} and Al^{Me} genes may be a result of natural selection although the evolutionary forces which maintain the trait are not known. A likely explanation is interbreeding between populations during several generations.

Neel (1973) estimated that the mutation rates for blood proteins in South American Indians could reach eight times those assumed for other populations, a feature attributable to a high degree of chromosome breakage, high serum mercury levels, extensive viral disease and the use of hallucinogens.

The influence of environmental factors on mutation rates is well demonstrated in Japan where the incidence of heterozygotes of 1:6000 in most regions of the country contrasts considerably with 1:400 in Hiroshima and Nagasaki.

CHAPTER 2

2.1 INTRODUCTION

Routine hospital cellulose acetate electrophoresis at pH 8.6 revealed two non-identical variant albumins of the slow type.

The first incidence was discovered at Redhill General Hospital, in the sera of an English mother and son living in the neighbourhood of Redhill, Surrey, U.K. The variant was designated albumin Redhill after the place of origin.

The second alloalbumin migrates more slowly than albumin Redhill at pH 8.6. It was identified in 1981 at Warwick Hospital in the serum of an unrelated Indian woman living in Warwick, U.K., and was termed albumin Warwick-2.

Fig. 2.1 shows the serum patterns of all three patients on polyacrylamide gel electrophoresis (PAGE) (Davis, 1964).

2.2 OBJECTIVES

Hereditary alloalbuminaemia is the rare occurrence of an amino acid substitution(s) in the primary sequence of normal serum albumin, giving rise to a second albumin having either a faster, slower or equivalent electrophoretic

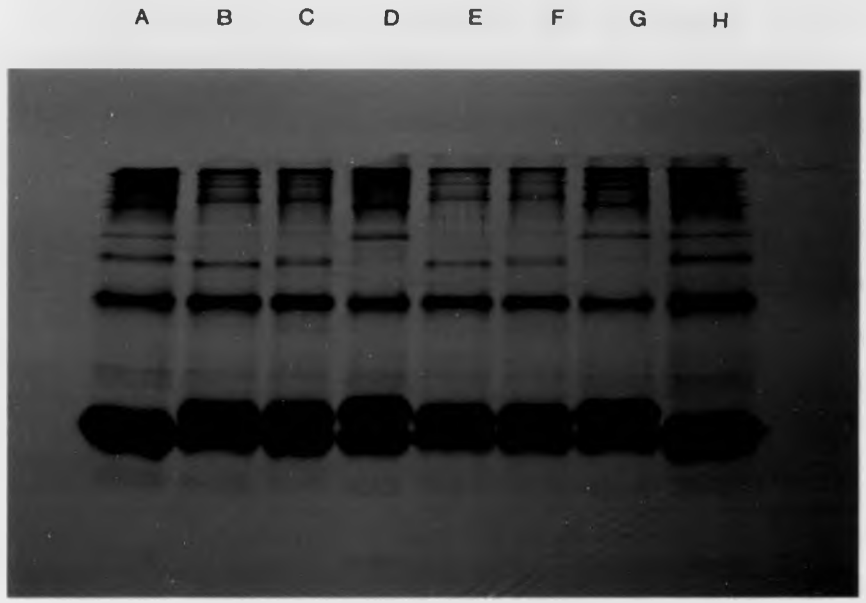


Fig. 2.1 Polyacrylamide gel electrophoresis of variant sera. A and H - normal serum, B, C, E and F- albumin Redhill, D and G- albumin Warwick-2.

mobility (Section 1.8.2).

The main objective of the research was to isolate the variant albumins, identify the aberrant amino acids and finally to locate the substitution in the primary structure.

2.3 EXPERIMENTAL STAGES

The research was performed in 6 stages. The initial step involved the characterisation of albumins Redhill and Warwick-2 using standard methods of classification (Section 1.8.2.7 and Section 1.8.2.8).

The second stage was the isolation of the variant albumins from serum, free from albumin A and other

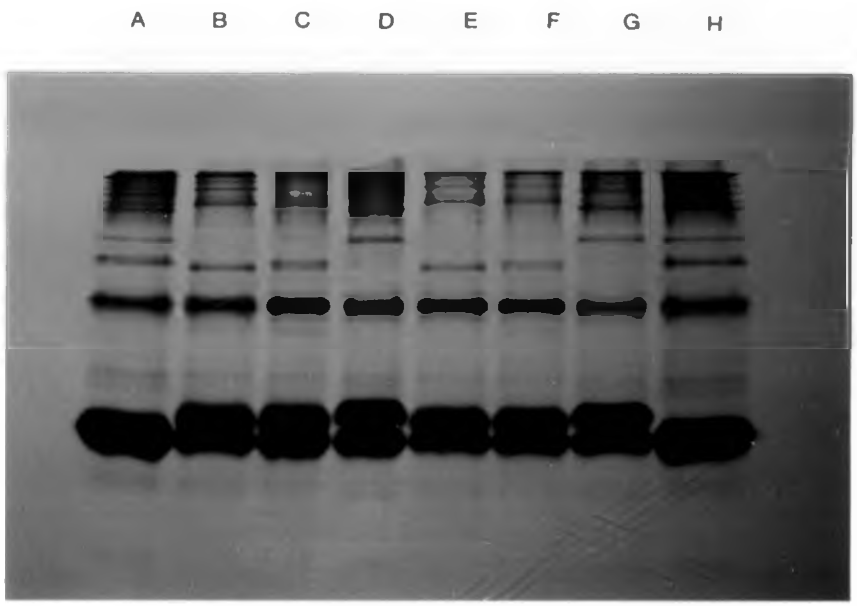


Fig. 2.1 Polyacrylamide gel electrophoresis of variant sera. A and H - normal serum, B, C, E and F- albumin Redhill, D and G- albumin Warwick-2.

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The second stage was the isolation of the variant albumins from serum, free from albumin A and other contaminants. This condition was stringent so that any anomalies observed while comparing peptide maps with those of normal HSA could be attributed solely to the abnormal peptides.

Sequence analysis in the third phase involved fragmentation of the variant albumins into relatively large and readily accountable peptides. To this end, the sites of cleavage were aimed at those amino acids which occur only rarely in HSA, namely the six methionines and the lone tryptophan.

The fourth stage was the identification of the novel peptides by peptide mapping. This technique enabled the localisation of the anomalous peptides within the sequence.

Stage five encompassed various ligand binding studies, employed either to confirm or to give further information regarding the position of the amino acid aberration.

The final stage was not completed due to insufficient time. It was to have involved amino acid sequencing of the purified abnormal peptides in order to obtain a positive identification of the mutant residues.

CHAPTER 3

CHARACTERISATION OF ALBUMINS REDHILL AND WARWICK-2

3.1 INTRODUCTION

The variant albumins were classified according to the following parameters:

(i) Total protein in the serum.

(ii) Total albumin in the serum.

(iii) Electrophoretic mobility on six media.

(iv) Ligand binding capacities of normal and variant albumins visualised on the same six media.

(v) Relative proportions of normal and variant albumins in the serum.

(vi) Thermal stability.

(vii) Resistance to freezing and thawing.

(viii) Molecular weight.

(ix) Detection of a possible pro-peptide.

(x) Isoelectric point.

(xi) Immunological character.

Dr. A. L. Tárnoky at the Royal Berkshire Hospital kindly classified albumins Redhill and Warwick-2 for us by carrying out the electrophoretic mobility and ligand binding tests.

3.2 EXPERIMENTAL

3.2.1 Determination of Total Protein in Serum

The total protein contained in the variant sera was measured according to the Biuret procedure of Gornall *et al.* (1949). The Biuret reagent comprised copper sulphate ($CuSO_4.5H_2O$) (1.5 g), sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$) (6.0 g), NaOH (10% w/v, 300 ml) and potassium iodide (KI) (1.0 g) per litre.

3.2.1.1 Preparation of Standards

The standard protein used was Sigma normal HSA, Fraction V, essentially free from fatty acids with a purity of 96-99%.

Biuret reagent (4.0 ml) was added to the albumin solutions (1.0 ml) containing 1-14 mg of protein. The latter figure was corrected for water content by spectrophotometric measurement at 280 nm and the actual weight of protein present calculated using

$$E_{280}^{1\%} = 5.31.$$

(Hunter and McDuffie, 1959)

3.2.1.2 Preparation of Serum Samples

The sera were diluted 1:20 with water and lipid was removed by adding diethyl ether (1 vol. ether: 3 vol. serum). After mixing for 30 seconds, the two layers were separated by centrifugation in sealed vessels. Lipid was removed into the organic phase and Biuret reagent was

Table 3.1 Standard conditions for the determination of electrophoretic mobility

Medium	Electrophoresis buffer	Current (mA)	Voltage (V)	Time
Cellulose acetate (78 x 150 mm) Shandon Celagram	Barbitone, Oxoid 0.1 M, pH 8.6	7.5		1 hr 20 mins
Helena Titan III Zip Zone Cellulose acetate plates 2 3/8" x 3"	Barbitone 0.05 M, pH 8.8		110	35 mins
Filter paper Whatman 3 MM (36 x 5 cm)	Barbitone 0.05 M, pH 8.6	3.5		7.5 hr
Disc-PAGE (7%, pH 8.3 → 8.9 → 8.3)	*tris* (0.05 M)-glycine (0.38 M) pH 8.3	2-3 mA/gel		
Agar (Oxoid, 1%) (8.5 x 8.5 cm)	Barbitone 0.05 M, pH 8.6		200	1.5 hr
Agarose (1%) (Corning ACI) sucrose (5%) EDTA (0.035%) in barbital (0.065 M, pH 8.6)	Barbitone 0.1 M, pH 8.6		90	45 mins

added to the clarified aqueous layer.

The absorption of standards and samples was measured at 540 nm after 30 minutes at room temperature.

3.2.2 Determination of Total Albumin in Serum

The total albumin present in both sera was evaluated using the specifically binding dye bromocresol green (Doumas *et al.*, 1971; Gustaffson, 1976).

Undiluted serum or standard normal HSA solutions (Section 3.2.1.1) (10 μl) were added to 2.0 ml of the dye solution: bromocresol green (0.15 mM), sodium succinate (75 mM), and 'Brij 35' surfactant (30% w/v, 4.0 ml) per litre, pH 4.20 $\pm$ 0.05.

Mixing and timing began simultaneously and the absorbance of the albumin-dye complex was monitored at 629 nm, commencing at 10 seconds after mixing and continuing for 2-3 minutes.

Albumin values were obtained by extrapolating the absorbance curves back to time = 0.

The standard solutions ranged from 1-10 g albumin/dl, corrected for water content.

3.2.3 Electrophoretic Mobility

Table 3.1 gives a summary of the conditions employed for the evaluation of electrophoretic mobility. The scheme originates from the Royal Berkshire Hospital test schecule (Tárnoky, 1980).

3.2.3.1 Staining

All electrophoretograms except for filter paper were stained with Ponceau S (0.2% w/v in TCA 3% w/v) and destained in acetic acid (7% v/v). The albumins were located on filter paper by drying the paper at 110°C for 10 minutes and then staining with bromophenol blue (1% w/v in 95% ethanol saturated with mercuric chloride), and destained in tap water.

3.2.4 Ligand Binding

For all electrophoretic methods except for disc-PAGE, serum (0.1 ml) was incubated with 50 μl of 0.05% aqueous solutions of each of the following dyes: bromophenol blue, bromocresol green, Ponceau S, Congo red, HABA and bilirubin, at 37°C for 10 minutes. The molar ratio of albumin:dye was approximately 2:1 except for HABA, in which case the ratio was 1:2.

For disc-PAGE, serum-sucrose (0.3 ml) was similarly incubated with 50 μl of each dye solution and the complete 350 μl loaded over the spacer gel.

The behaviour of the albumin-dye complexes was then observed by electrophoresis on the same six media as indicated in Table 3.1.

At the end of each run, the final appearance of the electrophoretograms was noted before staining. The number and positions of the albumin bands were marked and quantitative estimations of the extent of ligand binding were made visually. The positions of unbound ligand were also acknowledged. The patterns were then stained and the positions of the albumins confirmed after destaining.

3.2.5 Estimation of the Relative Proportions of Albumins in Serum

The albumins were separated by cellulose acetate electrophoresis in barbitone buffer (0.075 M, pH 8.6). Location of the bands was effected by extended staining in Ponceau S (0.9 g), TCA (13.4 g), sulphosalicylic acid (13.4 g)/litre to ensure complete penetration of the dye.

The background was destained in acetic acid (5% v/v) and the acid subsequently removed by thorough washing in distilled water.

The individual bands were excised and the dye eluted with NaOH (1.0 ml, 0.4 M) by agitation for several hours. The red colour was restored upon the addition of acetic acid (40%, 0.1 ml), and the cellulose acetate fragments were removed by centrifugation.

The absorbance of each eluate was measured at λ_{max} = 512 nm.

3.2.6 Tests of Stability

3.2.6.1 Thermal Stability (Arends *et al.*, 1969)

Sera were heated at 56°C for 30 minutes and 2 hours.

3.2.6.2 Resistance to Freezing and Thawing

(Izquierdo *et al.*, 1971)

Sera were twice frozen to -20°C and thawed at 37°C a few hours later. Sera were also frozen at -20°C, thawed at 37°C and immediately re-frozen, 10 times. In addition, rapid freezing and thawing was conducted 30-35 times over a time course of several days.

For both sets of tests, the resultant mobility and proportion of albumins Redhill and Warwick-2 were examined by cellulose acetate electrophoresis against the untreated variant sera.

3.2.7 Determination of Molecular Weight by SDS-PAGE

The molecular weight of normal and variant albumins was measured using isolated, defatted mixed albumins and sera diluted 10 times in water. As an aid to calculation, commercial molecular weight markers (BDH) spanning the range 12,300-78,000 were run alongside the albumin samples on each gel.

The gel system was based upon the discontinuous *tris*-buffer system of Laemmli (1970), employing a 12% separating gel containing *tris*-HCl (0.375 M), SDS (0.1% w/v), pH 8.9, and a 3% stacking gel, 0.125 M in *tris*-HCl and 0.1% in SDS, pH 6.8. This gel contained sucrose (40%) to increase mechanical strength. The electrophoresis buffer comprised *tris* (0.05 M), glycine (0.384 M) and SDS (0.1% w/v), final pH 8.3.

Samples were prepared for electrophoresis in the following buffer: *tris*-HCl (0.0625 M), glycerol (10% v/v), SDS (2% w/v), 2-mercaptoethanol (5% v/v) and bromophenol blue (0.01% w/v) as detailed in the manufacturers instructions (BDH Chemicals Ltd., England "Molecular weight markers for SDS-PAGE"). 10-20 μg of protein was applied per sample well and gels (16 x 16 x 0.15 cm) were electrophoresed at 60 V for 30 minutes, and then at 120 V until the tracker dye had reached the base of the gel (6-7 hours).

Gels were simultaneously fixed and stained in PAGE Blue G-90 (0.25% w/v in methanol (45% v/v) and acetic acid (10% v/v)). Destaining was performed at 40°C in methanol (5% v/v), acetic acid (7.5% v/v) (Weber *et al.*, 1972).

3.2.8 Detection of Proalbumins by Limited Tryptic Digestion

The possibility that albumins Redhill and Warwick-2 may have N-terminal propeptides was investigated by digestion with trypsin under mild conditions.

3.2.8.1 With Purified Mixed Albumins (Quinn *et al.*, 1975; Brennan and Carrell, 1980)

The mixed albumins (10-15 mg) were chromatographed on a column (1.5 x 62 cm) of Ultrogel AcA 34 eluted with sodium phosphate-HCl buffer (0.01 M, pH 7.5) containing NaCl (0.1 M). This procedure removed any contaminating α_2-macroglobulin which acts as a trypsin inhibitor (Brennan and Carrell, 1980). Elution was at 18 ml/hour and the albumin fractions were pooled, dialysed against distilled water at 4°C and lyophilised.

The mixed albumins (5 mg) were dissolved in sodium phosphate-HCl buffer (1.0 ml, 50 mM, pH 7.5) containing calcium chloride (50 μM). Trypsin-TPCK (N-tosyl-L-phenylalanyl chloromethyl ketone) (1 μg) was added and digestion followed for 1 hour at 22°C. The reaction was stopped with soya bean trypsin inhibitor (2 μg) and the solution fractionated on a column (1 x 43 cm) of Sephadex G-50 eluted with sodium phosphate-HCl (10 mM, pH 7.5) at a flow rate of 14 ml/hour. The albumin peak was dialysed against water and lyophilised. Any conversion of albumins Redhill or Warwick-2 into albumin A was assessed by non-denaturing disc-PAGE (Davis, 1964).

3.2.8.2 With Serum (Rousseaux *et al.*, 1982)

Trypsin-TPCK (10 μl, 5 mg/ml) in ammonium bicarbonate buffer (0.1 M, pH 8) containing calcium chloride (0.1 mM) was added to variant sera (100 μl) and the mixture incubated at 37°C for 2 hours, 4 hours and 6 hours. Digestion was stopped with soya bean trypsin inhibitor and cellulose acetate electrophoresis was carried out immediately at two pH levels: at pH 8.6 in barbitone buffer (0.075 M) and at pH 5.0 in sodium acetate buffer (0.031 M) containing EDTA (0.004 M) at 8 V/cm for 2-3 hours using untreated sera as standards.

3.2.9 Isoelectric Points

The isoelectric points of normal and abnormal albumins were determined by isoelectric focusing (IEF) either in gel tubes or in thin layers of gel on both polyacrylamide and agarose media. Approximate pI values were obtained by focusing the proteins in a broad pH range. Subsequently, a more accurate determination was possible in a narrow pH range.

3.2.9.1 Analytical IEF in Polyacrylamide Gel Tubes (T = 5%. C = 3%)

3.2.9.1.1 Focusing

The gels (9 x 0.6 cm i.d.) containing Pharmalyte pH 4-6.5, were prepared as detailed in the book entitled "Polyacrylamide Gel Electrophoresis", supplied by Pharmacia (Great Britain) Ltd. After prefocusing for 30 minutes at 1 mA/gel, non-defatted albumin (10-20 µg) was applied to each gel and focusing was continued at 1-2 mA/gel, constant current for 3.5-4 hours. All gels were water-cooled to about 10^{o}C.

The anode buffer consisted of (DL) glutamic acid (0.01 M) with L-histidine (0.01 M) at the cathode.

3.2.9.1.2 Fixing, Staining and Destaining

After focusing was complete, the proteins were fixed overnight in TCA (11.5% w/v) / sulphosalicylic acid (3.45% w/v) / methanol (30% v/v), and then stained in PAGE Blue G-90 (0.115% w/v) dissolved in destaining solution: ethanol (25%v/v)/acetic acid (8% v/v).

Destaining was at 40^{o}C.

3.2.9.1.3 Measurement of the pH Gradient

Commercially available pI markers (Pharmacia (Great Britain) Ltd.) were used for accurate isoelectric point determination. The particular set of markers applicable in this instance were contained in the Low pI Calibration Kit (pH 2.5-6.5) and were prepared according to the manufacturers' instructions. In the final, stained gels, four markers were visible: glucose oxidase (pI 4.15) soya bean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20) and bovine carbonic anhydrase B (pI 5.85).

3.2.9.2 Analytical IEF in Thin Layer Polyacrylamide Gels (T = 5%, C = 3%)

Gels were cast onto thin, silanised glass plates (25 x 12.5 cm). Pre-treatment of the glass with Silane A174 enables the gel to become chemically bonded to the plate by means of covalent linkages. Therefore, adhesion of the gel to the glass plate is ensured throughout the long fixing, staining and destaining processes. In addition, gel damage due to tearing and problems of gel distortion through shrinkage and swelling are minimised.

3.2.9.2.1 Focusing

The gels (11 x 11 x 0.15 cm) were cast by following a combination of instructions described in the book entitled "Isoelectric Focusing-Principles and

Methods" by Pharmacia, and in LKB Application Note 250. Gels incorporating the pH 3-10 range Pharmalyte were focused at 15 watts constant power with 25 mA maximum current and 1000 V maximum voltage. Under these conditions, the focusing time was 1.75 hours.

Gels containing the pH 4-6.5 range Pharmalyte were focused at 12 watts constant power with 15 mA maximum current and 750 V maximum voltage. Focusing was complete after 3 hours.

Before application of the samples, gels were prefocused for 30 minutes to remove persulphate and any contaminating acrylic acid which may cause gradient drift.

All gels were cooled to 10^{o}C.

The electrolytes were as follows:

pH Range	Anolyte (+)	Catholyte (-)
3-10	H_3PO_4 (0.1 M)	NaOH (1.0M)
4-6.5	(DL) glutamic acid (0.01 M)	L-histidine (0.01 M)

3.2.9.2.2 Fixing, Staining and Destaining

After completion of focusing, gels were fixed for 1 hour in TCA (10% w/v) / sulphosalicyclic acid (5% w/v). The gels were freed from ampholytes by immersing in destaining solution, methanol:acetic acid: water (3:1:6) for 30 minutes, after which time they were stained for 3-6 hours in PAGE Blue G-90 (0.2% w/v) dissolved in destaining solution. Destaining was at 40^{o}C.

3.2.9.2.3 Measurement of the pH Gradient

For both pH ranges, the Low pI Calibration Kit was used to measure the resultant gradient. In the pH 3-10 range, the following markers were visible: amyloglucosidase (pI 3.50), glucose oxidase (pI 4.15) soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), and human carbonic anhydrase B (pI 6.55).

3.2.9.3 Analytical Agarose IEF

3.2.9.3.1 Focusing

Thin layer agarose gels (11 x 11 x 0.15 cm) were prepared by adding Agarose IEF (0.1815 g) and sorbitol (99^{+}% pure) (2.178 g) to distilled water (16.34 ml) and heating at 100°C until dissolved. The solution was allowed to cool to about 70°C and Pharmalyte pH 3-10 or pH 4-6.5 (1.15 ml) was added. The gel was quickly cast into Gel Bond film as described by Pharmacia, and left for 1 hour at 4°C in a humidity chamber to harden.

Gels incorporating both pH ranges were focused at 7 watts constant power with 75 mA maximum current and 750 V maximum voltage. Focusing required 1.5 hours under these conditions at 10°C. The anode solution comprised H_2SO_4 (0.05 M) with NaOH (1.0 M) at the cathode, for both ranges.

The pH gradient was measured using pI standards as detailed for PAG-IEF.

Fixing, staining and destaining utilised the same solutions and followed the same sequence as for thin-layer PAG-IEF.

3.2.10 Immunological Character

The immunological properties of albumins Redhill and Warwick-2 were examined by two methods.

(i) The double immunodiffusion procedure of Öuchterlony.

(ii) Immunoelectrophoresis.

The extent of immunological similarity to albumin A was determined by reacting variant sera and purified normal and abnormal albumins with goat anti-albumin A.

3.2.10.1 Öuchterlony Double Diffusion (Öuchterlony, 1949)

3.2.10.1.1 Preparation of the Agar Gel (Clausen, 1981)

Agar Noble (electrophoretic grade) was de-fined in distilled water. NaCl (0.83% w/v) and sodium azide (0.1% w/v) in water were added to the agar (1.5% w/v). Dissolution was aided by heating at 100°C in a flask loosely stoppered by an inverted 5 ml volumetric flask.

Alcohol-washed microscope slides were covered with molten agar (4.0 ml) and left to set at room temperature.

Wells (10µl approximate volume) were cut in the agar with a truncated Pasteur pipette using a template for guidance, such that 8 wells were situated circularly around a central well. The radius of the circle was 7.5 mm and the well contents were removed with a needle.

3.2.10.1.2 Determination of a Balanced Antigen: Antibody System

Initial trials to fix a balanced proportion between antigen and antibody were done by performing immunodiffusion in 2 series:

(i) A fixed quantity of the antigen diffusing against a series of antibody dilutions. Pure albumin A (10 μl, 1 mg/ml) was applied to the centre well. Antiserum (10 μl) was applied to the surrounding wells after the following dilutions in water: undiluted, 1 in 2, 1 in 3, 1 in 4, 1 in 5, 1 in 10, 1 in 15 and 1 in 20.

(ii) A fixed quantity of the antibody diffusing against a series of antigen dilutions. Undiluted antiserum (10 μl) was applied to the centre well and pure albumin A (10 μl, 1 mg/ml) diluted in water as above, applied to the surrounding wells.

The slides were placed in a humidity chamber at $4^{o}C$ and diffusion was allowed to proceed for 16 hours.

3.2.10.1.3 Preservation of the Slides

The gels were washed for 24 hours in phosphate buffered saline, (0.05 M phosphate, pH 7.0) containing NaCl (0.8% w/v) to remove unreacted antigen and antibody and to stop diffusion. The agar was then washed free of NaCl with distilled water for several hours and the immunoprecipitates subsequently stained with

Amido Schwartz 10B (0.1% w/v in acetic acid (5.4% v/v), sodium acetate (0.72% w/v) and glycerol (1% v/v)) for 10 minutes. Slides were detained in acetic acid (5% v/v) for 2 hours.

Gels were dried by overlaying them with filter paper (Whatman 542) being careful to exclude air bubbles, and drying under vacuum at 37°C (about 12 hours) or 60°C (about 2 hours). Alternatively, if left open to the air at room temperature, complete drying required 48 hours.

3.2.10.1.4 Immunoprecipitation of Albumins Redhill and Warwick-2

The centre wells contained undiluted antiserum (10 µl) and surrounding wells contained solutions (10 µl, 1 mg/ml) of pure albumin A, pure albumins Redhill and Warwick-2, and pure albumin A isolated from the variant sera, all diluted 1 in 5. Diffusion, preservation and staining of slides was as previously described (Section 3.2.10.1.3).

3.2.10.2 Immunoelectrophoresis (Scheidegger, 1955)

3.2.10.2.1 Preparation of the Agar Gel (Clausen, 1981)

(i) For immunoelectrophoresis of serum

Agar noble (electrophoretic grade, 1% w/v) was dissolved in sodium veronal-HCl buffer (0.05 M, pH 8.4) containing sodium azide (0.1% w/v) by heating at 100°C. Alcohol-washed microscope slides were covered with the molten, buffered agar (2.0 ml) and set aside to harden.

Two wells (capacity about 5 μl) were cut in parallel approximately 2 cm from one end of the slide and filled with serum (2 μl). Electrophoresis was performed in sodium veronal-HCl buffer (0.05 M, pH 8.4) at 10 V/cm for 3.5 hours.

The slide was removed from the tank and a longitudinal channel of width 1 mm was cut equidistant between the sample wells, using two razor blades secured in parallel. Antiserum (40 μl) was spread in the channel and immunodiffusion was allowed to proceed at $4^{o}C$ in a humidity chamber for 16 hours.

(ii) For immunoelectrophoresis of pure variant albumins

4.0 ml of agar was poured per slide and solutions of the pure albumins Redhill and Warwick-2 (1 mg/ml, 15-20 μl) were used to fill more capacious wells cut as described previously. The proteins were electrophoresed in sodium veronal-HCl buffer (0.05 M, pH 8.4) at 10 V/cm for 6 hours. Antiserum (60 μl) was used to fill the central channel and immunodiffusion took place in a similar manner to that stated for serum.

3.2.10.2.2 Washing, staining and preservation of slides

Gels were washed and the immuno-precipitation lines stained following the procedures for Öuchterlony diffusion (Section 3.2.10.1.3). Slides were dried under vacuum at $37^{o}C$.

Slides of serum were also stained after the electrophoresis stage to show the extent of separation of the albumins before the diffusion step.

3.3 RESULTS AND DISCUSSION

The total concentration of serum proteins in the subject carrying albumin Redhill was 68 g/litre. This figure is well within the normal range of 60-80 g/litre. The total protein concentration of serum containing albumin Warwick-2 was considerably higher, at 102 g/litre.

The total albumin levels of both sera were within the normal range of 35-50 g/litre. Albumin A/albumin Redhill constituted 39 g/litre whereas albumin A/albumin Warwick-2 comprised 40 g/litre of serum. Figures 3.1 and 3.2 show the standard curves obtained for total protein (Biuret) and total albumin (bromocresol green) determinations respectively.

The relative proportion of albumin Redhill: albumin A in the serum is 40%:60% while albumin Warwick-2 constituted 45% of the total albumin against 55% of albumin A. Albumin Redhill, therefore, constitutes 15.6 g/litre with the level of albumin Warwick-2 at 18.0 g/litre of serum. Albumin variants usually comprise approximately half of the total serum albumin, with a slight preponderance of the normal allotype (Section 1.8.2.10.4). Albumin Warwick-2 also falls into this category but albumin Redhill may be slightly anomalous in this respect, with only 40% of the variant present. However, it is appreciated that these relative values are only approximations

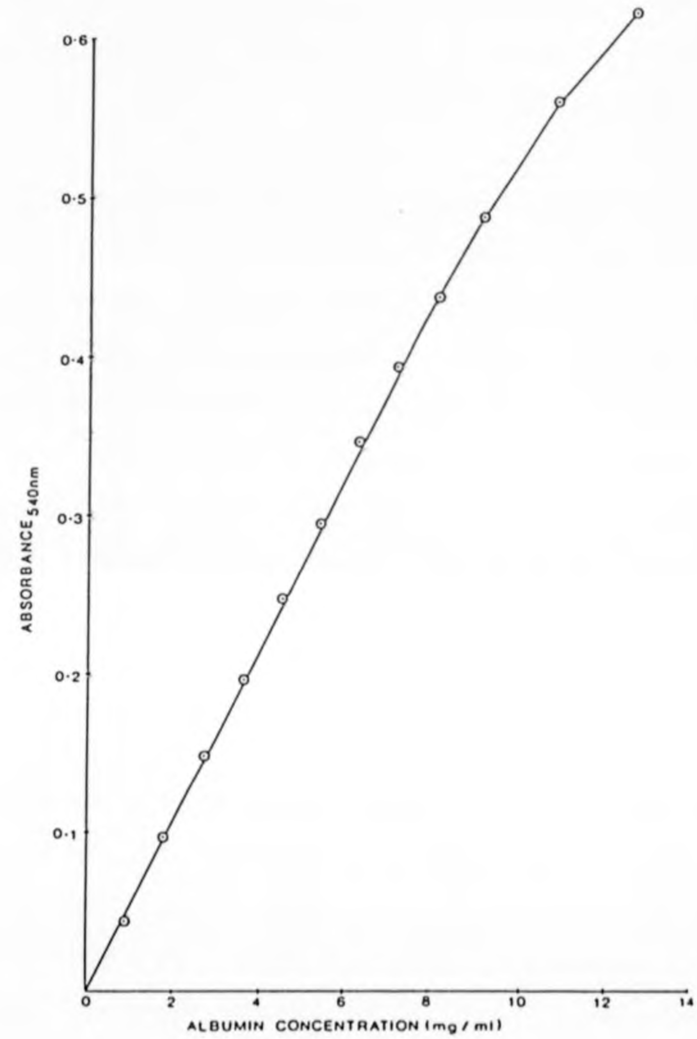

Fig. 3.1 Total protein: Biuret standard curve.

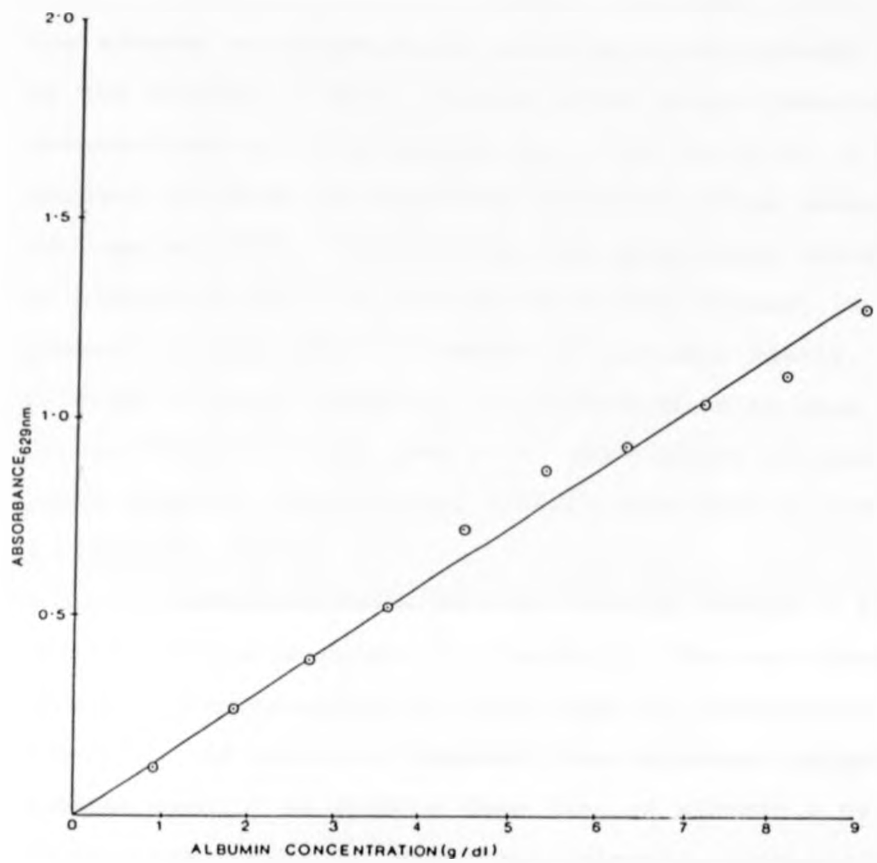

Fig. 3.2 Total albumin:bromocresol green standard curve.

since differential binding of Ponceau S by normal and variant albumins due to the altered primary structure of the variants, may obscure the true relationship between the concentrations of each albumin.

The relative mobilities on cellulose acetate electrophoresis, and the concentrations of both variants remained unchanged after repeated freezing and thawing and by heating at 56^{o}C. This shows that both albumins

Redhill and Warwick-2 are monomeric variants and that the altered electrophoretic mobility is not brought about by the binding of small ligands which should have been detached during these procedures. The character of the variant albumins was similarly unaltered after prolonged storage at -20°C. Furthermore, the hereditary nature of albumin Redhill is certain since this variant is present in more than one member of the same family. Although a closer familial investigation of albumin Warwick-2 has not been possible, the results of these tests strongly suggest that albumin Warwick-2 is not a transient variant.

SDS-PAGE revealed that albumin Warwick-2 has a similar molecular weight to albumin A. The two albumins cannot be distinguished on this basis by conventional SDS-PAGE. In contrast, however, the molecular weight of albumin Redhill is greater than that of albumin A by about 2,200. Fig. 3.3 shows the molecular weight gel of albumin A/albumin Redhill. This is very unusual since other monomeric variants, where this parameter has been measured, show no molecular weight differences to albumin A. Moreover, a substitution of only one amino acid by another would be unlikely to cause an identifiable change in mass since even the molecular weight of proalbumin, which has an additional hexapeptide, is indistinguishable from the molecular weight of albumin (Section 1.8.2.15). This indicates that albumin Redhill has undergone more than a single base change and has therefore not arisen merely as a result of a single amino

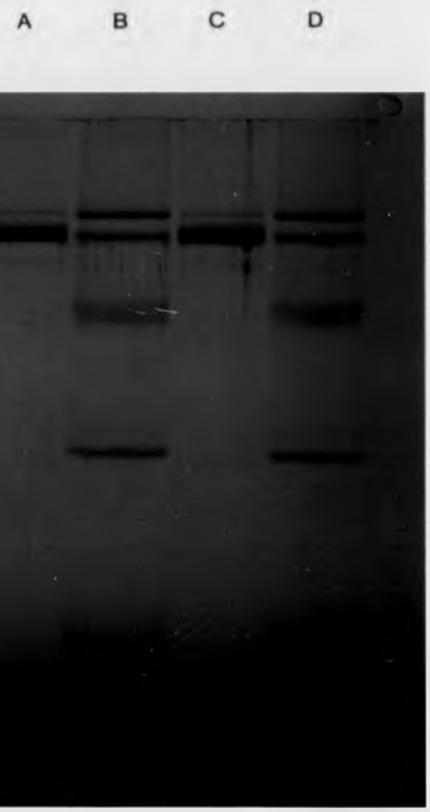

Fig. 3.3 Molecular weight determination of albumin Redhill by SDS-PAGE (Laemmli, 1970).
A and C, albumin A/albumin Redhill;
B and D, standard molecular weight markers.

acid mutation, whereas albumin Warwick-2 would appear to be a more 'conventional' variant. The molecular weights calculated using commercial standards are: albumin A, 65,400, albumin Warwick-2, 65,400, albumin Redhill, 67,600. Fig. 3.4 shows a sample standard molecular weight curve (albumin A). These calculations confirm that both variants are monomeric.

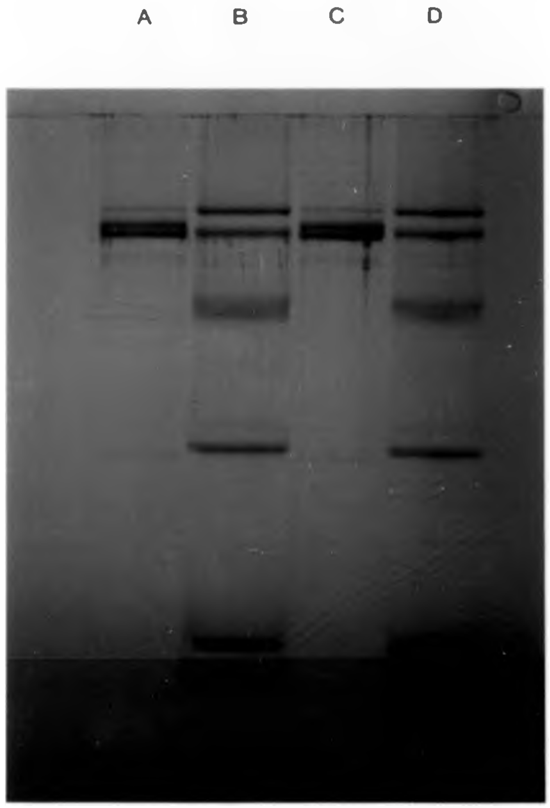

Fig. 3.3 Molecular weight determination of albumin Redhill by SDS-PAGE (Laemmli, 1970).
A and C, albumin A/albumin Redhill;
B and D, standard molecular weight markers.

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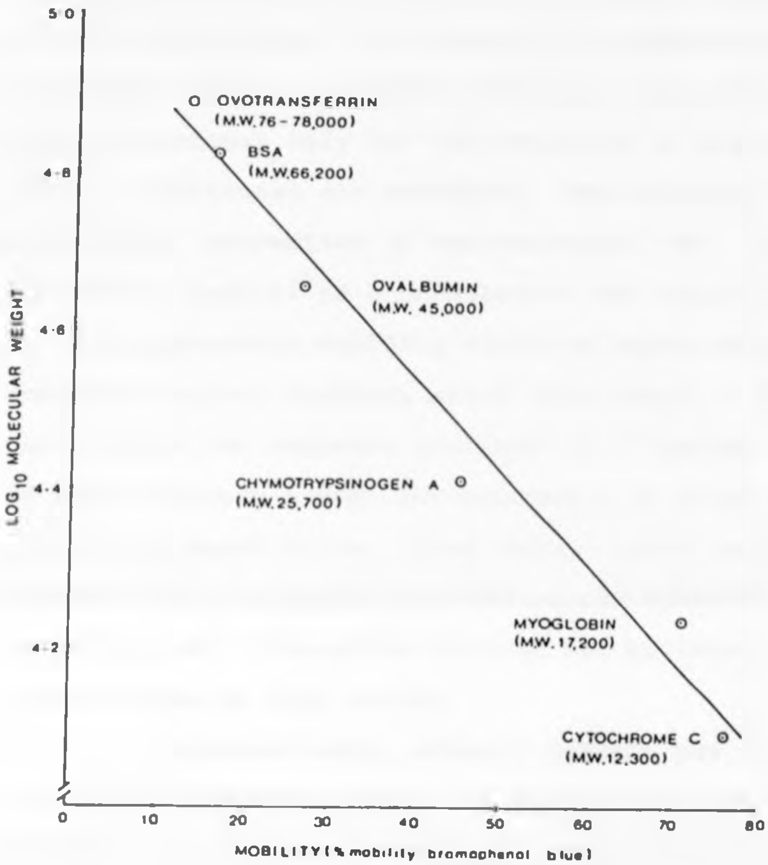

Fig. 3.4 Standard molecular weight curve (albumin A).

The method of limited tryptic digestion used to establish whether or not the variants are proalbumins proved to be inconclusive when applied to albumin Redhill. Digestion of both the serum and purified mixed albumins gave similar results. The electrophoretic mobility of the variant was unaltered at both pH 8.6 and 5.0. Albumin Redhill is indistinguishable from albumin A at pH 5.0, both before and after treatment with trypsin. However, the unchanged mobility cannot disprove that albumin Redhill is not a proalbumin: the degree of difference between the molecular weights of albumin Redhill and albumin A is too large to account only for the increase in size associated with an additional six residues; the molecular weight of the normal propeptide is approximately 880. Therefore, if albumin Redhill is a proalbumin, the expected change in electrophoretic mobility would be obscured by the remaining mutant residues which must exist at some other position in the sequence and lead to a further weight increase of approximately 1,300, corresponding to about ten to twelve additional amino acids. This change alone is likely to account for a retarded electrophoretic mobility. Consequently, an N-terminal propeptide cleaved off by trypsin may be undetectable by this method.

Alternatively, albumin Redhill may not be a proalbumin since the loss of a basic region of the sequence is likely to cause some change in electrophoretic mobility. However, further evidence obtained from metal binding studies (Chapter 7) and end group determination (Chapter 5) support the proposal that this variant is a proalbumin.

Albumin Warwick-2 is not a proalbumin. The electrophoretic mobility of the variant was unchanged at pH 8.6 and 5.0 after digestion with trypsin. Unlike albumin Redhill, albumin Warwick-2 is distinguishable from albumin A at pH 5.0, migrating more slowly at both pH levels. Since there is no uncharacteristic mutation to obscure the propeptide in this variant, it can be

Medium	pH Range	Isoelectric Point	
Polyacrylamide gel cylinders	4-6.5	Albumin A	4.93
		Albumin Redhill	4.75
		Albumin Warwick-2	5.02
Polyacrylamide thin layer gel	3-10	Albumin A	4.9
		Albumin Redhill	4.7
		Albumin Warwick-2	5.0
Polyacrylamide thin layer gel	4-6.5	Albumin A	4.80
		Albumin Redhill	4.74
		Albumin Warwick-2	5.0
Agarose thin layer gel	3-10	Albumin A	4.9
		Albumin Redhill	4.7
		Albumin Warwick-2	5.0
Agarose thin layer gel	4-6.5	Albumin A	4.83
		Albumin Redhill	4.75
		Albumin Warwick-2	5.0

Table 3.2 The isoelectric points of albumin A, albumin Redhill, and albumin Warwick-2

concluded that albumin Warwick-2 does not possess an additional N-terminal hexapeptide.

Under similar conditions, the pro-peptides of proalbumins Christchurch and Lille were cleaved and the electrophoretic mobility of the variants reverted to that of albumin A (Section 1.8.2.15).

The isoelectric points of albumins A, Redhill and Warwick-2 are detailed in Table 3.2.

Non-defatted albumin A showed one band and mixed albumin A/albumin Redhill and albumin A/albumin Warwick-2 showed two well-defined bands in gel cylinders in the pH range 4-6.5. In the pH 3-10 range on both polyacrylamide and agarose thin layer gels each albumin focused as a single band. However, in the pH 4-6.5 range on both media, an additional, minor band with a pI of 4.65 was present in all albumin samples. This extra band is probably due to the established heterogeneity of albumin preparations (Section 1.6) and is a manifestation of differential fatty acid binding by all the molecules in a sample. This low pI band is not visible in the pH 3-10 range which is too broad to be able to resolve the heterogeneous forms.

The pI values for albumin A obtained in gel cylinders are slightly higher than those calculated using the thin layer technique. This is probably due to the difficulty in reproducibly measuring and casting individual gels.

The pI of albumin Redhill is lower than the pI of albumin A, while albumin Warwick-2 has a higher pI.

Since both variants are of the slow type it was expected that their pI's would fall on the basic side of albumin A, since slow albumins are more positively charged and hence should require less protonation to reach electrical neutrality. While albumin Warwick-2 conforms to this pattern, albumin Redhill does not. Isoelectric focusing suggests that this variant has an overall more negative charge than albumin A whereas non-denaturing electrophoresis suggests a more positive net charge. A contributary factor to this anomalous behaviour may be the increased molecular size which retards the variant behind albumin A on conventional electrophoresis, obscuring an overall more negative charge than the usual allotype.

Fig. 3.5 shows the stained immunoprecipitates after Öuchterlony immunodiffusion. From preliminary investigations, the optimum conditions for the formation

Fig. 3.5 Öuchterlony immunodiffusion of albumins A, Redhill and Warwick-2. A, E, I and M, albumin A from pooled human serum; B, C and D, albumin A from serum containing albumin Redhill; F, G and H, albumin Redhill; J, K and L, albumin A from serum containing albumin Warwick-2; N, O and P, albumin Warwick-2.

of a precipitation line occurred between undiluted antiserum and albumin solutions (1 mg/ml) diluted

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of a precipitation line occurred between undiluted antiserum and albumin solutions (1 mg/ml) diluted

1 in 5. The immunoprecipitates fused completely in the central area indicating that both albumins Redhill and Warwick-2 possess a similar antigenic make-up to albumin A. The immunological behaviour of the variant albumins is indistinguishable from that of albumin A by this technique.

Fig. 3.6 shows the immunoelectrophoretic patterns of both variant sera. Gels stained after

(a) albumin Warwick - 2

albumin Redhill

(b) albumin Warwick - 2

albumin Redhill

Fig. 3.6 Immunoelectrophoresis of sera containing albumins Redhill and Warwick-2 after:
(a) electrophoresis only
(b) electrophoresis followed by immunodiffusion.

electrophoresis of serum revealed that albumin Warwick-2 was visibly distinct from albumin A whereas albumin Redhill and albumin A remained inseparable. The morphology of the immunoprecipitates associated with immunoelectrophoresis is influenced by the mobility of the antigens (Hirschfeld, 1960) so that for the albumin fraction, albumin A/ albumin Warwick-2 shows two arcs fused in the centre reminiscent of one antigen with two main electrophoretic mobilities but with similar immunological properties. The albumin fraction containing albumin Redhill shows only one arc located alongside the electrophoretic zone.

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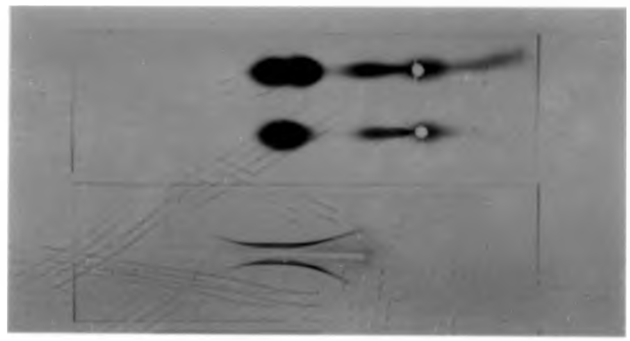

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Table 3.3 Relative mobility and ligand binding properties of albumins Redhill and Warwick-2 according to the RBH test schedule

Medium	Cellulose Acetate	Helena	Disc-PAGE	Agar	Agarose
Number of albumin bands visible					
Albumin Redhill	2	2	2	1	2
Albumin Warwick-2	2	2	2	2	2
Relative mobility (0-100 mm)					
Albumin A/Albumin Redhill	Incomplete separation	97-100, 94-96	92-100, 84-91	-	Incomplete separation
Albumin A/Albumin Warwick-2	100, 88	100, 91	94-100, 82-91	89-100, 78-87	94-100, 84-90
Dye Binding					
Bromophenol blue					
Albumin A/Albumin Redhill	A > S	A > S	A > S	-	A > S
Albumin A/Albumin Warwick-2	A > S	A > S	A > S	A > S	A > S
Bromocresol green					
Albumin A/Albumin Redhill	A > S	A > S	A = S	-	A > S
Albumin A/Albumin Warwick-2	A > S	A = S	A > S	A = S	O, O
Ponceau S					
Albumin A/Albumin Redhill	O, O	O, S	A > S	-	O, O
Albumin A/Albumin Warwick-2	O, O	O, O	A > S	O, O	O, O
Congo Red					
Albumin A/Albumin Redhill	O, O	O, O	A > S	-	A > S
Albumin A/Albumin Warwick-2	O, O	O, O	A > S	A = S	A = S
HABA					
Albumin A/Albumin Redhill	O, O	O, O	O, O	-	O, O
Albumin A/Albumin Warwick-2	-	-	O, O	-	-
Bilirubin					
Albumin A/Albumin Redhill	A > S	A > S	A > S	-	A > S
Albumin A/Albumin Warwick-2	A > S	A = S	A > S	A = S	A = S

<u>KEY</u>

A Albumin A

S Slow albumin variant (Albumin Redhill or Warwick-2)

O No observable binding

\- Not determined

Both pure albumins Redhill and Warwick-2 gave a single precipitation arc with anti-albumin A. Hence, immunoelectrophoresis confirms the antigenic similarity of both variants to albumin A. However, these seemingly complete immunogenic reactions cannot prove total antigenic similarity to albumin A since the methods and antibody preparations used may not be selective enough to detect minute changes in primary structure. Polyclonal antibodies like the goat anti-albumin A used in this study are heterogeneous since they consist of antibodies which cross react with different parts of the albumin molecule. Monoclonal antibodies are required if a single amino acid mutation is to be detected. Lapresle and Doyen (1983) have recently reported two monoclonal antibodies to HSA which can detect the glutamic acid$_{570}$ to lysine substitution in albumin B.

The electrophoretic mobility tests show that both albumins Redhill and Warwick-2 are slow variants. Albumin Warwick-2 migrates more slowly than albumin Redhill at pH 8.6. Table 3.3 shows the combined results from the relative mobility and ligand binding tests for both variants.

Both sets of tests distinguish albumin Redhill from four other European variants classified under similar conditions: albumin RIH (Chapman *et al.*, 1978), albumin Amsterdam (Sanders *et al.*, 1979)*, albumin Stirling and albumin Yorkshire (Curnow *et al.*, 1978)*. Albumin

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*Curnow, J. V., Fraser, G. P. and Tárnoky, A. L. (1978) *I.R.C.S. Med. Sci.*, 6, 525
Sanders, G. T. B., Curnow, J. V. and Tárnoky, A. L. (1979) *I.R.C.S. Med. Sci.*, 7, 581

Redhill migrates more closely to albumin A at pH 8.6 than any of these variants.

Comparing the dye binding properties, albumin Redhill binds bromophenol blue and Ponceau S similarly to albumin Amsterdam, bromophenol blue, Ponceau S, bilirubin and Congo red similarly to albumin Stirling, bromophenol blue, bilirubin and Congo red similarly to albumin Yorkshire but showed no common binding properties to albumin RIH on cellulose acetate, Helena, disc-PAGE and agarose. The other ligands are bound differently on one or more of the various media. However, it is not possible to make an absolute comparison since at the time of testing, the carrier of albumin Redhill was taking a variety of drugs and had previously undergone a succession of drug treatments over several years. Dye uptake by the albumins may be affected by the blocking action of bound drugs, as observed in albumins Stirling and Yorkshire.

Albumin Warwick-2 differs from several Indian variants available for comparison: albumin Warwick-1 (Tárnoky, private communication), albumin Jaffna (Bayliss *et al.*, 1983)*, albumin Vancouver (Frohlich *et al.*, 1978), albumin Birmingham (Bradwell *et al.*, 1975), albumin Kashmir (Tárnoky and Dowding, 1969), and albumin Hussain (Tárnoky, private communication). Albumin Warwick-2 binds bromophenol blue and Ponceau S similarly to albumin Jaffna, bromophenol blue similarly to albumins Warwick-1 and Kashmir but shows no common dye

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*Bayliss, V. M., Curnow, J. V., Tillyer, C. R. and Tárnoky, A. L. (1983) *I.R.C.S. Med. Sci.*, 11, 250-251 binding characteristics to albumins Vancouver and Hussain on cellulose acetate, Helena, disc-PAGE, agar and agarose media. The relative electrophoretic mobility is slower than any of these variants on all five media except for a faster mobility than albumin Vancouver on Helena and agarose, and than albumin Hussain on agarose.

Table 4.1 Some methods used in the purification of albumin variants

Variant	Separation Method	Final Purity	Reference
Albumin B	Starch block electrophoresis veronal buffer (0.1 M, pH 8.6)	85-95%	Gitlin *et al.* (1961)
Albumin Oliphant	DEAE-Sephadex sodium phosphate (0.2 M, pH 5.75)	> 95%	Winter *et al.* (1972)
Albumin Gainesville	DEAE-Sephadex sodium phosphate (0.15 M, pH 5.75)		Lapresle (1977)
Albumin Birmingham	DEAE-Cellulose sodium phosphate (0.02 M, pH 7.0) incorporating a linear salt gradient		Bradwell *et al.* (1975)
Albumin Kashmir	Chromatofocusing pH range 4-5	90%	Tillyer *et al.* (1982)
Proalbumin Christ-church	DEAE-Sephadex sodium acetate (0.025 M, pH 5.1-4.6)		Brennan and Carrell (1978)
Proalbumin Lille	Preparative cellulose acetate electrophoresis *tris*-veronal buffer (pH 8.6)	> 95%	Abdo *et al.* (1981)
Un-named fast and slow variants	QAE-Sephadex A-50 sodium acetate (0.1 M, pH 5.1-4.6)		Bradley and Hornbeck (1974)
Transient (pancreatitis)	DEAE-Sephadex A-50 sodium phosphate (0.4 M, pH 5.75)		Rousseaux *et al.* (1976)
Transient (penicillin)	DEAE-Sephadex sodium phosphate (0.15 M, pH 5.75)		Lapresle and Wal (1979)

CHAPTER 4

PURIFICATION OF ALBUMINS REDHILL AND WARWICK-2

4.1 INTRODUCTION

Albumin variants, for the most part have almost identical molecular weights to albumin A, as well as similar immunological properties. The only obvious means by which they differ from their normal counterpart are by charge and isoelectric points. The latter two properties have been exploited in various forms by workers wishing to isolate pure samples of either fast or slow alloalbumins in order to carry out subsequent structural investigations. To date, separations have involved ion-exchange chromatography, preparative electrophoresis and chromatofocusing. Table 4.1 summarises the methods used to purify some albumin variants.

Samples of pure albumin Redhill were obtained both by chromatofocusing and preparative polyacrylamide gel electrophoresis. Albumin Warwick-2 was purified exclusively by preparative-PAGE, since this latter method proved to be less time consuming and less expensive although no preference is expressed over the final preparation resulting from either technique.

Albumin A was purified from pooled human serum, the final stage being either chromatofocusing or dye-affinity chromatography. The initial purification stages for all the albumins followed the same route, namely, ammonium sulphate precipitation and gel filtration.

4.1.1 Chromatofocusing

4.1.1.1 Introduction

Chromatofocusing is a relatively new technique. A pH gradient is generated on an ion-exchange resin by taking advantage of the buffering action of the charged groups within it. Proteins are separated according to their isoelectric points.

The method gives high resolution, has a high capacity and is easy to operate.

4.1.1.2 Principle

The pH interval is chosen such that the isoelectric points fall roughly in the middle of the ensuing pH gradient. The appropriate Polybuffer exchanger is equilibrated with the starting buffer, the pH of which is set slightly above the upper limit of the pH gradient, whilst the pH of the eluant, Polybuffer, is adjusted to the pH value chosen for the lower limit. The pH gradient forms automatically as the charged groups on the exchanger are titrated with the large number of differently charged species which comprise the eluant, and the proteins emerge from the column in order of decreasing isoelectric point.

4.1.1.3 Behaviour of Proteins on the Column

A protein migrating down the column is

continually attached and detached from the ion-exchanger depending on the pH of the environment and the charge on the protein at any particular instant.

When the pH of the eluting buffer is below the pI of the protein, the protein is positively charged and so does not bind to the anion exchanger. The pH of the buffer increases with distance from the point of origin and when the changing pH rises above the pI, further down the column, the protein reverses its charge and becomes bound to the exchanger, where it remains until the gradient causes the pH to fall below the pI, releasing the protein again. This process is repeated throughout the length of the column until the protein emerges at its pI.

4.1.1.4 The Focusing Effect

A protein applied to the column migrates in the eluant as far as its isoelectric point, whereupon it travels more slowly until it elutes. If a similar protein sample is applied some time after the first sample, it descends at the same speed as the eluant until it catches up with the slower-moving sample. The proteins then proceed down the column at the same speed until they co-elute. This effect increases the resolution of the method.

4.2 EXPERIMENTAL

4.2.1 Ammonium Sulphate Precipitation (McMenemy *et al.*, 1971)

Blood was collected from patients without the addition of anticoagulant. A crude preparation of albumin was first obtained by adding solid ammonium sulphate to sera with slow stirring at 0°C until 45% saturated (277 g/litre, pH 6.5). After about 20 minutes, the precipitate was removed by centrifugation at 7,000 r.p.m. at 4°C for 30 minutes. The ammonium sulphate saturation of the supernatant was increased to 75% (516 g/litre, pH 4.5) and the precipitate spun down at 15,000-18,000 r.p.m. for 30-60 minutes at 4°C.

4.2.2 Gel Filtration and Desalting

The second precipitate was retained and redissolved in the minimum volume of *tris*-HCl buffer (0.01 M, pH 7.0) containing NaCl (0.05 M). A suitable volume of this solution (the product from about 5-6 ml of original serum) was applied to a column (1.5 x 95 cm) of Sephadex G-100 equilibrated with the *tris*-buffer previously described. The column was eluted with the same buffer at a flow rate of 15 ml/hr. The albumin closely followed a turbid region of high lipid content and was easily recognisable owing to the yellow colour of protein-bound bilirubin. The albumin fractions were pooled, dialysed exhaustively against distilled water at 4°C and lyophilised.

It was convenient at this point to defat the albumin although chromatofocusing experiments were performed using the non-defatted protein. Albumins were defatted before preparative PAGE.

4.2.3 Defatting (Chen, 1967)

The charcoal treatment of Chen is a very mild technique and the native conformation of proteins remains undisturbed. Other bound ligands, particularly bilirubin are also removed.

Activated charcoal was washed with distilled water, filtered by suction and allowed to dry at room temperature.

Albumin was dissolved at room temperature in distilled water and charcoal (half the weight of protein) was mixed in. The pH of the solution was lowered to pH 3.0 with HCl (0.1 M) and the mixture stirred magnetically for 1 hour on an ice bath. The charcoal was removed by centrifugation at $2^{o}C$ and the clarified solution brought to neutrality by the addition of NaOH (0.1 M).

The defatted albumin was dialysed against distilled water at $4^{o}C$ and lyophilised

4.2.4 Chromatofocusing

4.2.4.1 Separation of the Albumins

The chosen pH range was pH 6-4. All gels and buffers were degassed before use to prevent irregularities in the pH gradient caused by dissolved atmospheric CO_2. This phenomenon occurs between pH 5.5 and 6.5 and may cause a pH plateau in this region.

A column (1 x 26 cm) of Polybuffer exchanger

(PBE) 94 was equilibrated with the starting buffer, histidine-HCl (0.025 M, pH 6.2). The column packing was checked by following the progress of cytochrome C (horse heart type IIA) which has a strongly basic pI.

A small volume of eluting buffer, Polybuffer 74-HCl (5-10 ml, 0.0094 mmol/pH unit/ml, pH 4.0) was allowed to run into the column prior to sample application to begin the gradient. The albumin samples (non-defatted, salt-free), either Albumin A or combined albumin A/ albumin Redhill (25 mg) dissolved in the starting buffer (3.0 ml), were applied to the column. Elution was at 10 ml/hour and fraction volumes were small (1.65 ml). At least 9 column volumes of eluant were required to generate the complete gradient.

4.2.4.2 Removal of Polybuffer

The individual albumin peaks were lyophilised and freed from Polybuffer by gel filtration on Sephadex G-100 in *tris*-HCl buffer (0.01 M, pH 7.0) containing NaCl (0.05 M). The albumin fractions were then dialysed against distilled water at 4°C and lyophilised.

Albumin Redhill eluted after albumin A during chromatofocusing. The relevant peak was rechromatographed under similar conditions to remove contaminating albumin A.

In later experiments, it was more productive to use large PBE 94 columns (1 x 40 cm) to enable greater quantities of albumin to be processed (up to 100 mg).

Also, to save time, the dialysis step

required after removing the Polybuffer from albumins separated by the first round of chromatofocusing was replaced by direct buffer exchange from *tris*-HCl/NaCl to histidine-HCl on Sephadex G-25 before the second separation.

4.2.5 Preparative Polyacrylamide Gel Electrophoresis

4.2.5.1 Separation of the Albumins

The mixed albumins were separated in non-denaturing slab gels (16 x 16 x 0.3 cm) (Davis, 1964, Laemmli, 1970), using a discontinuous *tris*-buffer system.

The separating gel (T = 7.5%, C = 2.7%) contained *tris*-HCl (0.375 M, pH 8.9) and the stacking gel (T = 3%, C = 2.7%), *tris*-HCl (0.125 M, pH 6.8). The gels were pre-electrophoresed for 30 minutes at 60 V constant voltage using *tris*-HCl (0.05 M, pH 8.3) as electrolyte, to remove persulphate ions from the immediate environment of the proteins.

Mixed albumin A/albumin Redhill (10 mg) or albumin A/albumin Warwick-2 (15 mg) were applied to the gels dissolved in a sucrose solution and the albumins were separated by electrophoresis at 60 V for 1 hour followed by 120 V constant voltage at 4^{o}C with *tris*-(0.05 M)-glycine (0.38 M), final pH 8.3, as the electrode buffer.

An additional small well was made alongside the main sample well in each gel in order to apply a small volume of bromophenol blue in sucrose solution, necessary for following the progress of electrophoresis. Albumin Warwick-2 was better separated from albumin A than albumin Redhill, therefore it was possible to apply a greater weight of this protein per gel .

4.2.5.2 Location of the Albumin Bands

In order to avoid binding unwanted ligands, the following detection method was devised.

After separation, the gels were removed from the glass plates and transferred onto clingfilm. The gel was placed directly over a fluorescent silica gel TLC plate (Kieselgel 60 F_{254}, Merck, Darmstadt). Viewing directly under ultra-violet light, the albumins showed up as dark bands on a fluorescent background and were excised with a razor blade.

4.2.5.3 Electrophoretic Elution of the Albumins (Otto and Šnejdárková, 1981; Mendel-Hartvig, 1982)

The elution gel consisted of a support gel in running buffer, the gel strip to be eluted (sample gel) and a stacking gel.

The support gel comprised acrylamide (T = 7.5%, C = 2.7%) in *tris*-(0.05 M)-glycine (0.38 M), pH 8.3. Pre-electrophoresis was carried out for 3 hours at 120 V before use. The sample gel was placed on top of the support gel and fixed in position by overlaying with a stacking gel (T = 3%, C = 2.7%) containing *tris*-HCl (0.125M, pH 6.8) approximately 2 cm in height. Reverse polarity electrophoresis was at 20 mA constant current. Under these conditions, the albumin concentrated in and eluted through the stacking gel, and with no staining was readily visible as a sharp, refractile line.

Just before the albumin left the gel, an overlay (3-4 ml) of glycerol (50% v/v) in stacking gel buffer was added which in turn was overlain with NaCl (2.0 M). Electrophoresis was continued until elution into the glycerol layer was complete, after which time, the NaCl was removed and the albumin-containing layer was dialysed exhaustively against distilled water at 4^{o}C and lyophilised.

4.2.6 Analysis of the Albumin Preparations

The purity of the final albumin preparations was determined by:

(i) Cellulose Acetate Electrophoresis
Electrophoresis was carried out at 8 V/cm in barbitone buffer (0.075 M, pH 8.6) for 2 hours. Electrophoretograms were stained in Ponceau S and destained in dilute acetic acid (Section 3.2.5).

(ii) Non-denaturing PAGE (Davis, 1964)
Analytical disc-PAGE without SDS was used to determine the extent of contamination by albumin A. The separating gel was 7.5% (C = 2.7%) and the stacking gel, 3% (C = 2.7%) in acrylamide.

(iii) Denaturing PAGE (Weber and Osborn, 1975)
SDS-disc PAGE was used to evaluate the overall purity of the albumins. The acrylamide concentrations in the separating and stacking gels were as for non-denaturing PAGE. Gels and electrophoresis buffer contained 0.1% SDS.

4.2.7 Purification of Albumin A by Dye-affinity Chromatography

Blue Sepharose CL-6B contains the dye Cibacron Blue F3G-A which selectively removes albumin from solution.

The gel was regenerated by washing cycles of alternate high and low pH. The column (1.9 x 24.5 cm) was washed with 10 column volumes of *tris*-HCl buffer (0.1 M, pH 8.5) containing NaCl (0.5 M), followed by 10 column volumes of sodium acetate buffer (0.1 M, pH 4.5) containing NaCl (0.5M) and then re-equilibrated with eluting buffer, *tris*-HCl (0.05 M, pH 7.0) containing NaCl (0.1 M).

Impure albumin A was dissolved in eluant and applied to the column at a very slow flow rate. Elution was ceased for 2 hours to facilitate binding to the dye. Unbound components were then removed by washing with 10 column volumes of eluting buffer and the albumin subsequently desorped with *tris*-HCl (0.05 M, pH 7.0) containing NaCl (1.5 M). The albumin fraction was dialysed against distilled water at 4^{o}C and lyophilised.

Purity was assessed by SDS-disc PAGE (Weber and Osborn, 1975).

4.3 RESULTS AND DISCUSSION

All sera gave a similar elution profile during gel filtration on Sephadex G-100 of the fraction obtained after precipitation with 75% saturated ammonium sulphate. A typical example is shown in Fig. 4.1 which denotes the fractionation of albumin Redhill from an original 6.2 ml of serum. The small peak eluting after albumin contained large quantities of ammonium sulphate as detected by barium sulphate precipitation.

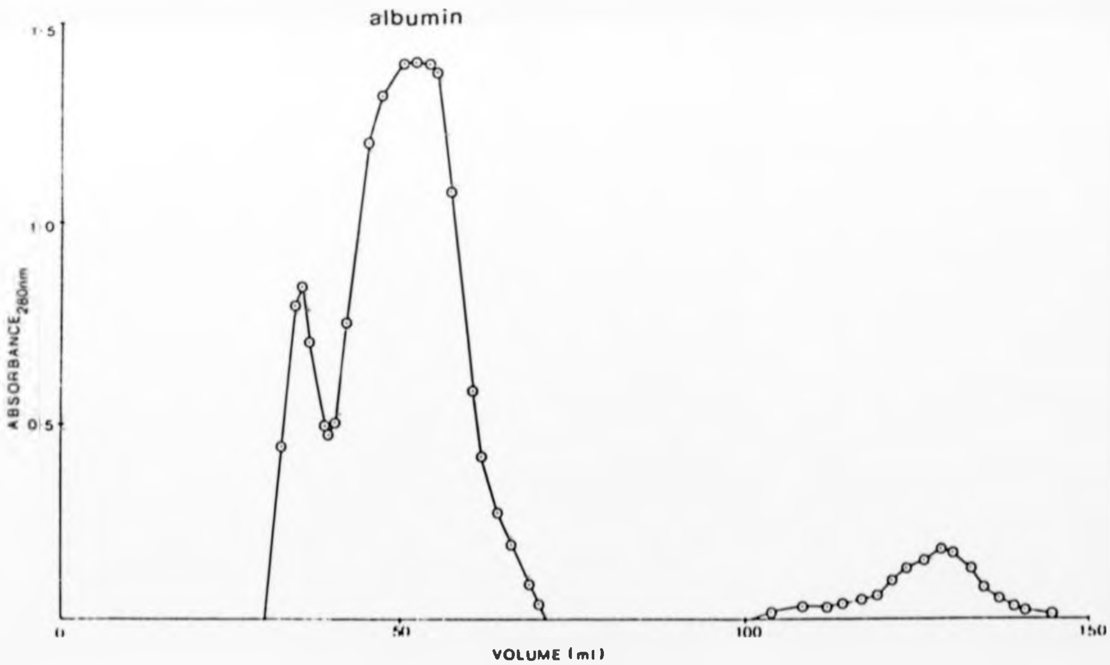

Fig. 4.1 Gel filtration of albumin Redhill on Sephadex G-100. Eluant is *tris*-HCl (0.01 M, pH 7.0) containing NaCl (0.05 M).

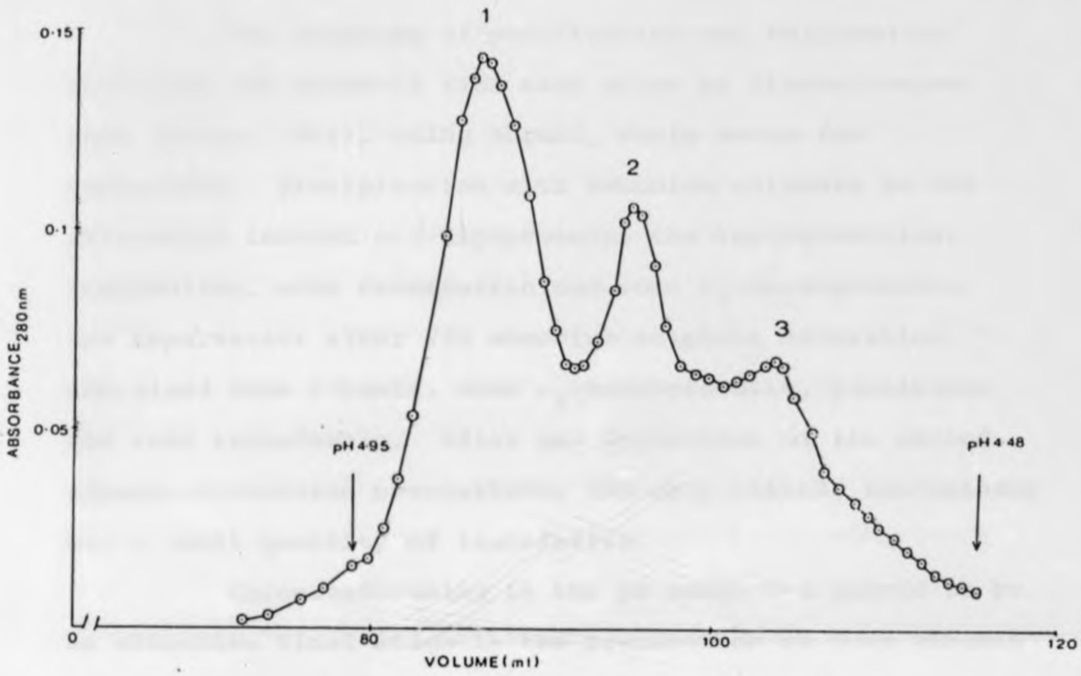

Fig. 4.2 Chromatofocusing of albumin A/albumin Redhill in the pH range 6-4.

Fig. 4.3 Analysis of the albumin peaks after chromatofocusing of albumin A/albumin Redhill, by disc-PAGE (Davis, 1964). A - peak 3; B - peak 2; C - peak 1; D - normal HSA.

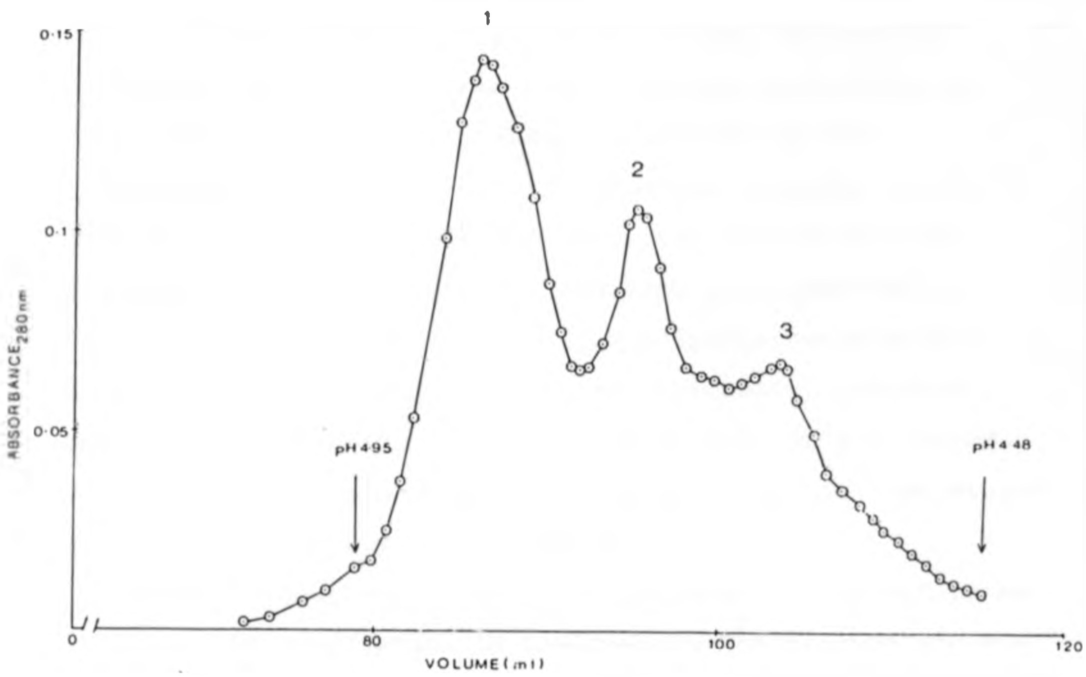

Fig. 4.2 Chromatofocusing of albumin A/albumin Redhill in the pH range 6-4.

Fig. 4.3 Analysis of the albumin peaks after chromatofocusing of albumin A/albumin Redhill, by disc-PAGE (Davis, 1964). A - peak 3; B - peak 2; C - peak 1; D - normal HSA.

The progress of purification was followed by analysing the products from each stage by discontinuous-PAGE (Davis, 1964), using normal, whole serum for comparison. Precipitation with ammonium sulphate at 45% saturation removed β-1-lipoprotein, the haptoglobulins, γ-globulins, some transferrin and some α_2-macroglobulin. The supernatant after 75% ammonium sulphate saturation contained some albumin, some α_2-macroglobulin, prealbumin and some transferrin. After gel filtration of the second, albumin-containing precipitate, the only visible contaminant was a small quantity of transferrin.

Chromatofocusing in the pH range 6-4 proved to be an effective final stage in the preparation of pure albumin Redhill. Fig. 4.2 shows the elution profile after the first fractionation by this technique. The mixed albumins eluted as three well-defined peaks although baseline separation was not achieved. Analytical isoelectric focusing (Section 3.2.9) demonstrated that albumin Redhill has a more acidic isoelectric point than albumin A and, in agreement with this, albumin Redhill eluted after albumin A from the chromatofocusing column.

Fig. 4.3 shows disc-PAGE analysis of the three peaks obtained after the first round of chromatofocusing of albumin Redhill. Normal HSA was used as a standard. Peak 1 contained mostly albumin A with a small quantity of albumin Redhill. Peak 2 was an almost equal mixture of albumin Redhill and albumin A but with a slight preponderance of the variant albumin, whereas the last peak consisted almost exclusively of albumin Redhill with a trace of albumin A. All fractions were contaminated slightly with transferrin.

Following the removal of Polybuffer and salts, the albumin Redhill fraction underwent a second separation to remove residual albumin A. The elution pattern again showed three peaks, and although resolution was rather poor, disc-PAGE analysis of the final peak revealed the pure variant. Gels showed no visible contamination with albumin A or transferrin.

Although this method finally yielded the variant albumin in a very pure form, its quantitative efficiency was rather low with only 25% recovery of the pure protein being attained. Washing the PBE column with strong salt solution indicated that the albumins had not been retained on the column and there are no other sources where appreciable losses would occur.

Evaluation of the analytical gels and the elution profiles indicates that the major factor responsible for the low yield is that the technique does not discriminate sufficiently between the isoelectric points of the two albumins to allow complete separation. Although some albumin Redhill elutes as a separate peak, the remainder always remains unresolved from albumin A. Hence, the overall process is not so much a complete separation, but a gradual "leaching out" of the variant albumin from the original mixed sample. Isolating large quantities of abnormal albumin by this technique would prove costly and time consuming although only very mild conditions are involved in the procedure.

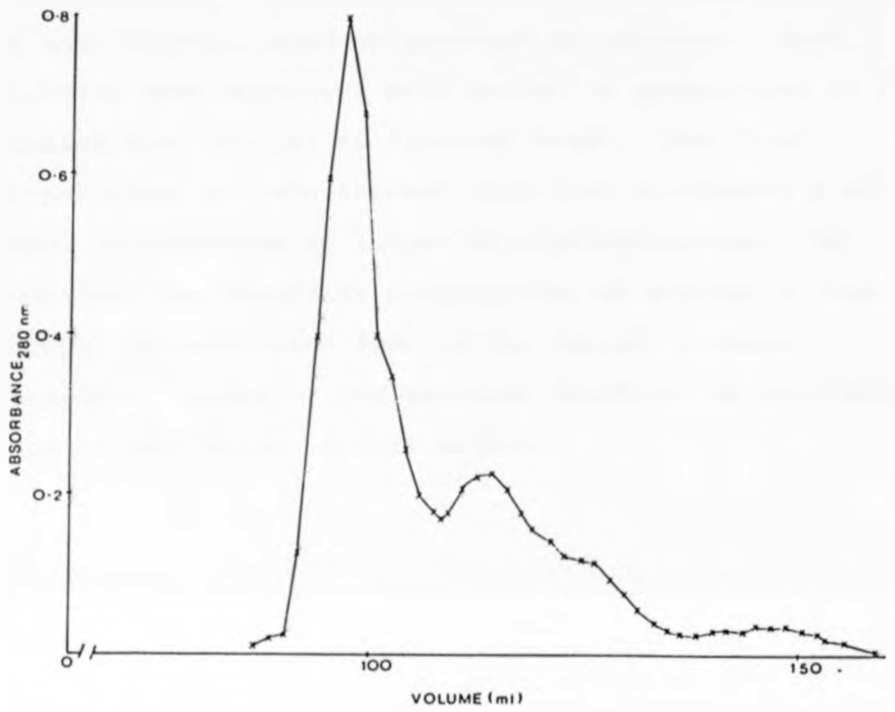

Fig. 4.4 Purification of normal HSA by chromatofocusing in the pH range 6-4.

Figure 4.4 shows the elution profile of normal albumin after chromatofocusing. Electrophoresis revealed the albumin to be pure and free from transferrin (pI 5.9). Purification of albumin A by affinity chromatography using Cibacron Blue F3G-A gave a similarly pure albumin preparation. Albumin eluted as a single peak.

Preparative PAGE was the most efficient method for purifying both albumins Redhill and Warwick-2 rapidly and cheaply and in high yield. During the electrophoresis stage, albumin Redhill did not separate from albumin A as well as albumin Warwick-2, so that less of this variant could be purified at one time. Both variants were separated well enough to enable them to be excised from the gel as distinct bands. The final preparations of each variant were free of albumin A and other contaminants as judged by electrophoresis. In addition, the resultant preparations of albumin A from each of the sera were free of the variant albumin. Therefore, losses of the variants could not be attributed to lack of resolution in this method.

CHAPTER 5

FRAGMENTATION STUDIES ON ALBUMINS REDHILL AND WARWICK-2

5.1 INTRODUCTION

This chapter describes the methods used for determining the N- and C-terminal end groups, and for chemical fragmentation of albumins Redhill and Warwick-2 at the methionine and tryptophan residues.

The N-terminal residue was identified by labelling with dansyl chloride. The C-terminal residue was determined by reaction with carboxypeptidase Y.

The methionine-specific reagent, cyanogen bromide and the tryptophan-specific o-iodosobenzoic acid and N-chlorosuccinimide were used to cleave the albumins into relatively large peptides. Before fragmentation, albumin was defatted and denatured, either by oxidation with performic acid or by reduction and S-carboxymethylation, except for reaction with carboxypeptidase and N-chlorosuccinimide where native albumin was used.

The fragmentation patterns were analysed by polyacrylamide gel electrophoresis, the details of which are described in the following chapter.

5.1.1 Performic Acid Oxidation (Hirs, 1967; Croft and Waley, 1971)

This procedure was used to denature albumin prior to reaction with dansyl chloride. The resultant protein is resistant to cleavage by CNBr since methionine is oxidised to methionine sulphone. Cysteine and cystine are converted to cysteic acid while tryptophan undergoes multiple transformations leading to the formation of N-formylkynurenine in which the indole ring is opened. The destruction of tryptophan prevents the use of tryptophan-specific reagents for fragmentation studies.

A large excess of reagent (at least 10-fold) is required. This is removed under mild conditions to prevent over-oxidation of the protein (phenolic and hydroxyl groups).

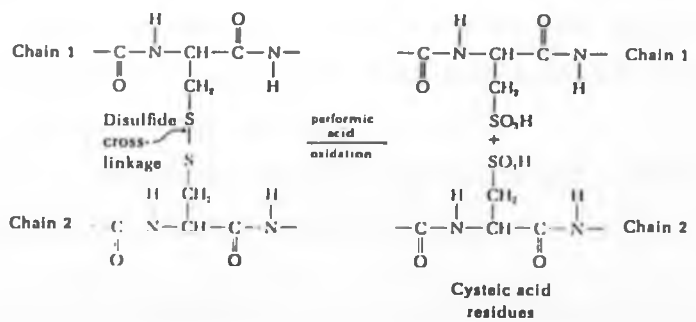

Fig. 5.1 Oxidative cleavage of disulphide cross-linkages by performic acid.

5.1.2 <u>Reduction and S-Carboxymethylation</u>

Disulphide bond reduction followed by blocking of the sulphydryl groups is a commonly used denaturing procedure which does not usually lead to the destruction of amino acids. The most satisfacotry reducing agents are β-mercaptoethanol (Hirs, 1967) or dithiothreitol/ dithioerythritol (Cleland, 1964). The reduction is performed in the absence of oxygen with a large excess of reagent at alkaline pH. Denaturants such as urea or guanidinium chloride ensure complete unfolding of the protein. Carbamylation of amino groups by ammonium cyanate which accumulates in aqueous solutions of urea is minimised by the use of amines for pH control, particularly *tris* and methylamine. Cyanate reacts more favourably with these buffer amines.

The sulphydryl groups were blocked with iodo-acetic acid. Excess reagent may modify other amino acids, namely methionine, histidine, lysine and tyrosine. S-carboxymethylation increases the negative charge on the protein (Gurd, 1972).

Reduced, alkylated proteins are generally insoluble in dilute, aqueous solutions.

$$\text{Protein–SH} + \underset{\text{iodoacetic acid}}{ICH_2COOH} \longrightarrow \text{Protein–S–}CH_2COOH + HI$$

Fig. 5.2 S-carboxymethylation of cysteine with iodoacetic acid.

5.1.3 Determination of the N-Terminal Residue

Several methods have been documented for the determination of the amino terminal residues in peptides and proteins including reactions with fluorodinitrobenzene (Sanger, 1945), phenylisothiocyanate (Edman, 1950), cyanate (Stark, 1972a), dansyl chloride (Gray, 1972) and 4-dimethylaminoazobenzene-4'-isothiocyanate (Dabitc)(Chang *et al.*, 1976). Enzymic digestion with leucine aminopeptidase and aminopeptidase M have also been used (Light, 1972).

The N-terminal residues of albumins Redhill and Warwick-2 were identified using 'dansyl' (5-dimethyl-aminonaphthalene-1-sulphonyl) chloride, (DNS-Cl), chosen due to the simplicity of methodology and high sensitivity. The limits of detection exceed 10^{-5} μmoles of DNS-amino acid using the polyamide layer system of identification and only 1-5 nmoles of original protein are required.

DNS-Cl reacts with N-terminal primary and secondary amines. Reactive side chain functions include thiol (cysteine), phenolic hydroxyl (tyrosine), amino (lysine) and imidazole (histidine) in decreasing order of reactivity. Amino groups only react as the free base ($R\text{-}NH_2$) and not as the conjugate acid ($R\text{-}NH_3^+$) so that successful labelling only occurs at alkaline pH, usually between pH 9 and 11. Excess DNS-Cl is hydrolysed to 1-dimethylaminonaphthalene-5-sulphonic acid (DNS-OH, dansic acid) by water and hydroxyl ions this being visible as a large blue fluorescent spot on the chromatogram.

5.1.3.1 <u>Principle of the Method</u>

The protein amino groups are labelled with DNS-Cl to give yellow/green fluorescent sulphonamide derivatives followed by hydrolysis of the peptide bonds and identification of the fluorescent DNS-amino acid derived from the N-terminus.

Dansyl derivatives are generally stable to hydrolysis. Cysteine and histidine sulphonamides are degraded to DNS-OH and the original group and the hydrolysis time must be limited if DNS-proline is suspected. DNS-serine, DNS-threonine and DNS-cysteine break down with the elimination of $DNS-NH_2$ (1-dimethyl-aminonaphthalene-1-sulphonamide) and DNS-tryptophan is destroyed.

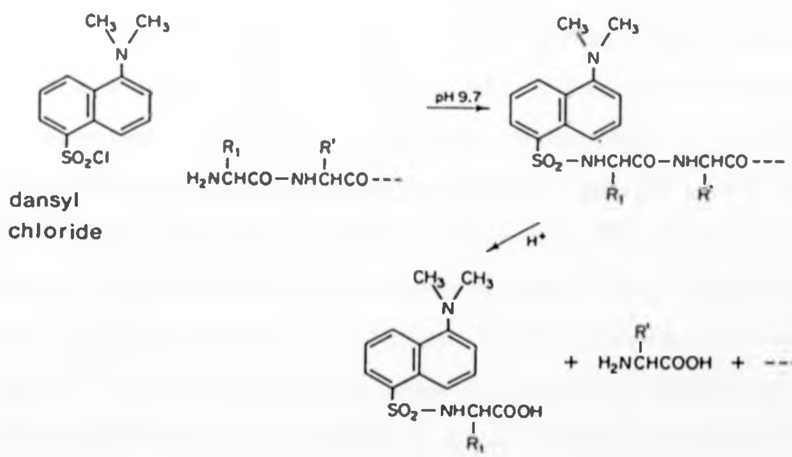

Fig. 5.3 Labelling the N-terminal residue of a protein with dansyl chloride.

5.1.4 Determination of the C-Terminal Residue

The methods available for C-terminal end group identification are more limited and less satisfactory than those for the determination of N-terminal residues. Some of the methods in use include hydrazinolysis (Schroeder, 1972), C-terminal selective tritium labelling (Matsuo *et al.*, 1966), 'subtractive' C-terminal determination (Parham and Loudon, 1978), hydantoin formation (Stark, 1972b) and digestion with carboxypeptidases A and B (Ambler, 1972), C (Tschesche, 1977) or Y (Hayashi, 1977).

The method of choice was partial hydrolysis with carboxypeptidase Y due to its convenience, reliability and specificity. Carboxypeptidase reacts with peptides and proteins to release amino acids possessing a free α-carboxyl group.

Carboxypeptidase Y is isolated from bakers yeast and has a broad specificity. All C-terminal amino acids are removed, including proline. Hydrophobic residues are released more rapidly than charged residues. Glycine in the penultimate position slows the release of the terminal amino acid. The enzyme does not usually react with native proteins, although albumin denaturation was found to be unnecessary. The C-terminal sequence is evaluated either by studying the kinetic release of amino acids after enzyme addition, or by thin layer chromatography.

5.1.5 Cleavage at Methionine with CNBr

Cyanogen bromide was introduced by Gross and Witkop (1961) and has proved to be the most useful chemical protein cleavage reagent available. It has the unique specificity of breaking peptide bonds on the carboxyl side of methionine residues. Since methionine occurs relatively rarely in proteins, albumin has only 6 such residues, the resulting fragments are usually large and consequently relatively easy to manipulate. These peptides are also valuable in the ordering of small peptides from enzymic digests.

The reaction is performed in strong acid at room temperature with an excess of reagent. Under these conditions, methylthiocyanate is formed and methionines are converted to a mixture of homoserine and homoserine lactone, the two being interconvertible (Ambler, 1965).

In albumin, the met-cys bond at met_{123} is only partially susceptible to cleavage (Doyen and Lapresle, 1979). Methionine is converted to homoserine without bond breakage. Taking this into account, eight peptides were expected from cleavage of albumin.

Cystine residues are unaffected by CNBr in acid and their prior reduction is unnecessary. Oxidation of methionine to the sulphone or sulphoxide prevents reaction with CNBr. Methionine sulphoxide results from autooxidation during protein or peptide isolation and can be remedied by pre-treatment with thiols (Westhead, 1972; Neumann, 1972).

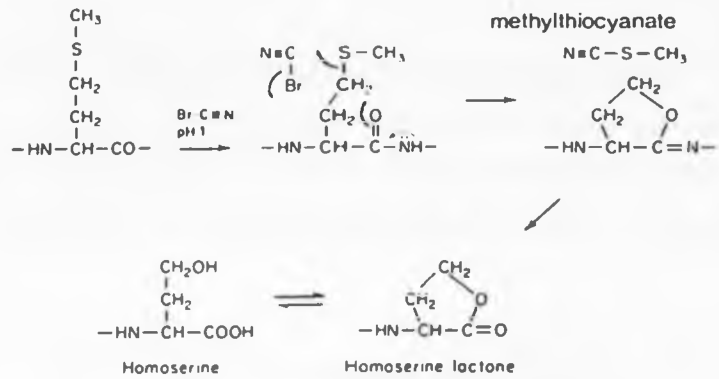

Fig. 5.4   Reaction of CNBr with methionine residues.

5.1.6   Cleavage at Tryptophan

HSA has a single tryptophan residue at position 214 in the sequence. Cleavage at this point yields two peptides, readily separable due to large size differences. The approximate molecular weight of the resultant N-terminal peptide is 24,000 and that of the C-terminal peptide, about 42,000. Cleavage at tryptophan was carried out for two reasons. Firstly, peptide analysis provided confirming evidence regarding the region of mutation and secondly, isolation of the abnormal peptide followed by further degradation would facilitate a more exact location of the amino acid substitution.

The cleavage reagents employed for this purpose were o-iodosobenzoic acid and N-chlorosuccinimide (NCS), both of which cleaved selectively at tryptophan. However, yields were low and reaction with o-iodosobenzoic acid proved to be non-reproducible.

5.1.6.1 Reaction with o-Iodosobenzoic Acid

o-Iodosobenzoic acid cleaves on the carboxyl side of tryptophan (Mahoney and Hermodson, 1979; 1980). The proposed reaction mechanism is shown in Fig. 5.5.

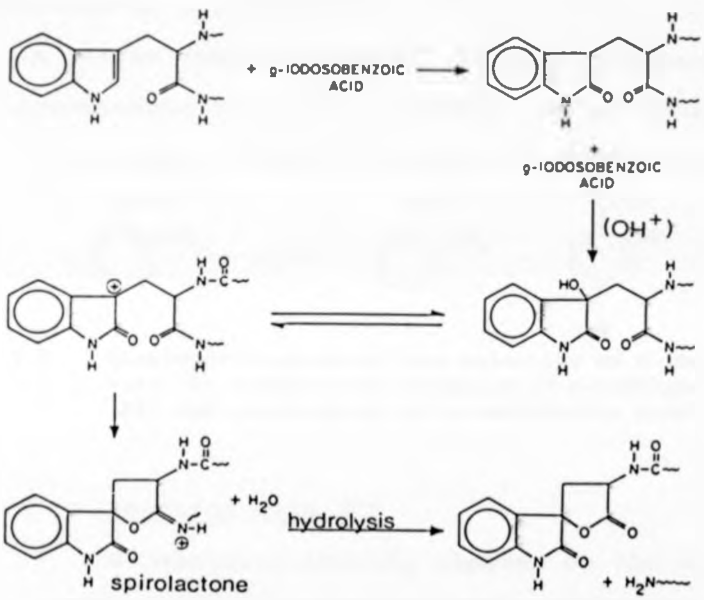

Fig. 5.5 Proposed reaction mechanism of o-iodosobenzoic acid at tryptophan.

The first oxidative step leads to the formation of the oxindole derivative. This reaction can occur under mild oxidative conditions and is therefore probably fairly rapid (Savige and Fontana, 1977). The second oxidation is rate limiting and is followed by a cyclisation of the carbonyl oxygen on the indole nucleus to form an iminospirolactone which hydrolyses to the C-terminal

N-acyldioxindolylalanine, so cleaving the peptide chain.

Side reactions are limited to the oxidation of methionine to methionine sulphoxide and possible cleavage of tyrosyl bonds. Modification and cleavage of tyrosine residues has been attributed to o-iodoxybenzoic acid (Fig. 5.6), a disproportionation product of o-iodosobenzoic acid identified as a contaminant present in most preparations of the latter (Mahoney *et al.*, 1981). Pre-incubation of o-iodosobenzoic acid with p-cresol causes reduction of the contaminant, thereby minimising tyrosine modification.

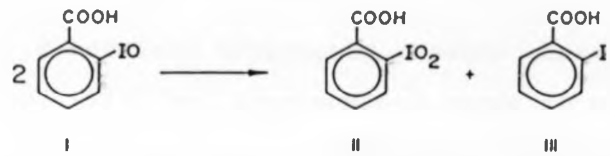

Fig. 5.6 Disproportionation of two molecules of o-iodosobenzoic acid (I) leads to one molecule of o-iodoxybenzoic acid (II) and one molecule of o-iodobenzoic acid (III).

5.1.6.2 Reaction with NCS

N-chlorosuccinimide cleaves on the carboxyl side of tryptophan by mild oxidative chlorination of the indole nucleus (Schechter *et al.*, 1976). A possible reaction mechanism is shown in Fig. 5.7.

In aqueous solution under weakly acidic conditions (pH 2-5), NCS oxidises the exposed indole rings. Two equivalents of active chlorine (Cl^+) halogenate the indole nucleus which then undergoes spontaneous dehalogenation through a series of oxidations and hydrolyses, leading to formation of the oxindole. This structure promotes bond cleavage.

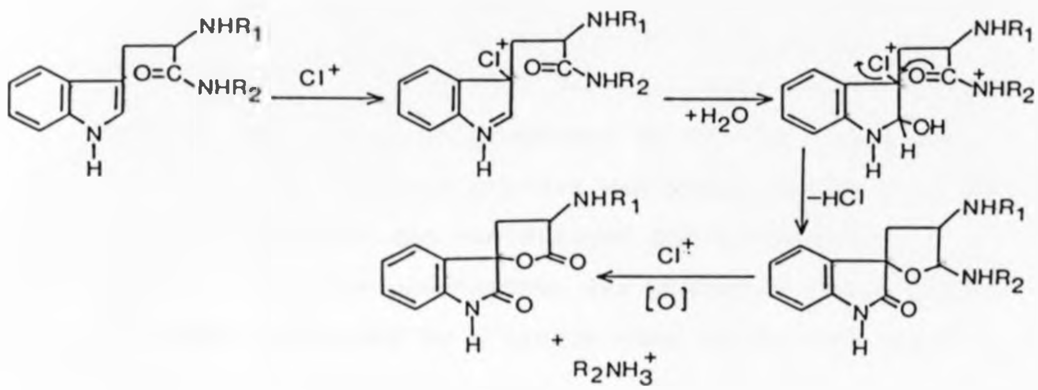

Fig. 5.7 Reaction at tryptophan by NCS.

N-terminal tryptophan resists cleavage (Wilcheck and Witkop, 1967) and tryptophanyl bonds in native proteins are generally inaccessible to this reagent. Methionine is oxidised to the sulphoxide and cysteine to cystine. Otherwise, other residues remain unaffected and no other peptide bond is cleaved.

5.2 EXPERIMENTAL

5.2.1 Performic Acid Oxidation

The performic acid reagent was prepared by combining hydrogen peroxide (2 vol., 30%) with formic acid (8 vol., 99%) and leaving to stand in a sealed container for 2 hours at 25^{o}C. The proportion of performic acid reaches a maximum at about this time and decreases slowly thereafter. The reagent was then cooled on ice for 15 minutes.

Albumin (1-2 mg) was dissolved in formic acid (0.5 ml, 99%) and cooled on ice for 15 minutes. The performic acid reagent (0.5 ml) was added to the protein solution and oxidation proceeded at $0^{o}C$ for 2.5 hours.

The oxidised protein was precipitated with TCA (1.5 ml, 25% w/v) and centrifuged for 5 minutes at 5,000 r.p.m. The precipitate was washed in three changes of ethanol followed by a single wash in diethyl ether before being dried *in vacuo*.

5.2.2 Reduction and S-Carboxymethylation (Allen, 1980)

Albumin (1-20 mg/ml) was dissolved in *tris*-HCl (0.1 M, pH 8.3) containing urea (8 M, deionised and recrystallised) and EDTA (1 mM). Dithiothreitol was added to a final concentration of 2 mM. Nitrogen (oxygen-free by bubbling through alkaline pyrogallol) was passed though the solution and incubation followed at $37^{o}C$ for 1 hour.

Sufficient iodoacetic acid (50 mM, neutralised with NaOH) was added to give a 1.1-fold molar ratio over the total protein thiol groups. The vessel was flushed with nitrogen and sealed, and alkylation took place in the dark at $37^{o}C$ for 1 hour. A more complete reaction was obtained by repeated addition of dithiothreitol (1 mM) followed by incubation at $37^{o}C$ for 1 hour, and then re-addition of iodoacetic acid (2 mM) followed by further incubation at $37^{o}C$ for 30 minutes.

2-Mercaptoethanol (1% v/v) was then added to remove excess iodoacetic acid and the solution dialysed exhaustively against ammonium bicarbonate (50 mM) containing thiodiglycol (0.01% v/v) before lyophilisation.

5.2.3 Determination of the N-Terminal Residue

5.2.3.1 Labelling with Dansyl Chloride (Gray, 1972)

Residual acid and possible traces of ammonia in the albumin samples were removed by dissolving the performic acid oxidised protein (50-250 μg) in ammonium bicarbonate (100 μl, 0.2 M) containing SDS (0.1% w/v) followed by lyophilisation. The sample was redissolved in SDS (50 μl, 1% w/v) by heating at $100^{o}C$ for 2-5 minutes. After cooling, N-ethylmorpholine (50 μl) was added with thorough mixing and the pH of the solution checked with pH paper. Dansyl chloride (75 μl, 25 mg/ml in anhydrous dimethylformamide) was introduced and any precipitating protein redissolved by adding a small volume of SDS (1% w/v). Dansylation proceeded for at least 1 hour at room temperature away from light.

The labelled protein was precipitated with dry acetone (0.5 ml) and the mixture centrifuged for a few minutes at 5,000 r.p.m. The compressed precipitate was washed once with acetone (0.5 ml, 80% v/v), centrifuged again and finally dried *in vacuo*.

5.2.3.2 Hydrolysis

The dried sample was transferred to a small glass test tube (50 mm x 4 mm i.d.) which had been pre-cleaned by overnight heating at 500°C. The peptide bonds were hydrolysed in HCl (100 μl, 6.1 M) for 16 hours at 105°C after flame-sealing the tube. After hydrolysis, the tube was opened and the HCl removed *in vacuo* over NaOH.

5.2.3.3 Identification of DNS-Amino Acids

The dansyl derivatives were solubilised in acetone: acetic acid (10 μl, 3:2 v/v) and chromatographed on 2-dimensional TLC on 5 x 5 cm double-sided polyamide plates using a solvent system based on that described by Woods and Wang (1967). The sample was applied to one corner of the plate and the chromatogram developed in the first direction with water: 90% formic acid (200:3 v/v). The plate was dried, rotated 90°C and run in the second solvent, toluene:acetic acid (9:1 v/v). For further clarification, a repeat run in the second direction using ethyl acetate:methanol:acetic acid (20:1:1 v/v/v) followed by ammonia (1.0 M):ethanol (1:1 v/v) was required.

Control DNS-amino acids, namely DNS-L-aspartic acid, DNS-L-arginine, DNS-L-glutamic acid, DNS-ε-L-lysine, di-DNS-L-lysine and N,O-di-DNS-L-tyrosine were similarly chromatographed on the reverse side of each plate and the spots were visualised under u.v. light at 365 nm.

5.2.4 Determination of the C-Terminal Residue

5.2.4.1 Reaction with Carboxypeptidase Y (Lee and Riordan, 1978)

The enzymic activity (12.5 μmol/mg protein at pH 6.75, 25°C) of carboxypeptidase Y (5 μg) was restored by incubating at 0°C overnight in water (20 μl). The water was added pre-cooled to 0°C. The endopeptidase activity of contaminating yeast proteinase A was selectively inhibited by pre-incubation with pepstatin A (7 μg) in 2-(N-morpholino)-ethane sulphonic acid (MES) buffer (20 μl, 10 mM, pH 6.8) for 2 minutes at room temperature.

Native albumin (0.5 mg) was dissolved in MES buffer (200 μl, 10 mM, pH 6.8) and added to the activated enzyme. Digestion was allowed to proceed for 10 minutes at 37°C, after which time the reaction was stopped by freezing and the digest lyophilised. A control sample comprised albumin A without added enzyme.

5.2.4.2 Identification of the C-Terminal Residue

The C-terminal residue was identified by ascending TLC on silica gel plates using chloroform: methanol:30% ammonia (2:1:1 v/v/v) as the solvent system. After drying, the chromatogram was sprayed with ninhydrin: acetic acid reagent (0.2% ninhydrin w/v in 95% ethanol: acetic acid, 20:1 v/v) and the amino acids visualised by heating at 60°C for 30 minutes.

The amino acid standards L-leucine and L-glycine were chromatographed alongside the albumins for comparison. These amino acids form the terminal and penultimate residues of albumin A.

5.2.5 Cleavage at Methionine

5.2.5.1 Reaction with Cyanogen Bromide (Allen, 1980)

Normal and mixed albumins were dissolved (5 mg/ml) in aqueous formic acid (70% v/v). Solid CNBr (a weight equal to that of protein dissolved in a small volume of formic acid (70% v/v)) was added and the solution stirred magnetically for 20-22 hours in the dark at room temperature (typically 21°C) under oxygen-free nitrogen. The mixture was then diluted with water (15 vol.) and lyophilised. For complete removal of acid and by-products, dilution with water followed by lyophilisation was repeated.

The fragments (5 mg/300 µl) were reduced in triethanolamine-acetate buffer (0.2 M, pH 8.6) containing urea (8 M), methylamine (25 mM, cyanate trap), dithiothreitol (60 mM) and EDTA (10 mM) for 4 hours at 37°C (Franklin *et al.*, 1980). The peptides were then alkylated to prevent the formation of mixed disulphides, by adding iodoacetic acid (130 mM, neutralised with NaOH) and reacting for 30 minutes at room temperature in the dark. The reaction was stopped by the addition of excess dithiothreitol.

The samples were diluted with water (5 vol.) and precipitated by adding TCA to 20% w/v. The fragments were collected by centrifugation, washed with acetone: HCl (0.2% w/v) followed by acetone and dried *in vacuo*. All solutions contained 2-mercaptoethanol (1% v/v) during precipitation.

Peptide analysis is described in Chapter 6.

5.2.6 Cleavage at Tryptophan

5.2.6.1 Reaction with o-Iodosobenzoic Acid

Reduced and S-carboxymethylated albumin (5 mg) was dissolved in a minimum volume of urea (8 M)/dithiothreitol (20 mM)/thiodiglycol (1% v/v) by incubation at 37°C for about 30 minutes. o-Iodosobenzoic acid (1 mg/100 μl) dissolved in acetic acid (80% v/v)/urea (8 M) was pre-incubated with p-cresol (0.1 mole p-cresol/mole o-iodosobenzoic acid) at room temperature in the dark for at least 2 hours, and was then added to the albumin in a volume equivalent to twice the weight of protein. Reaction took place at room temperature in the dark for 24 hours and was stopped by adding excess dithiothreitol (5 mg). The solution was dialysed against acetic acid (10% v/v) in the dark at 4°C and lyophilised.

Normal and mixed albumins with no added o-iodosobenzoic acid formed the control samples.

Peptide analysis is described in Chapter 6.

5.2.6.2 Reaction with N-Chlorosuccinimide (Shechter *et al.*, 1976)

Normal and mixed albumins (1 mg, 1.5×10^{-8} mol) were dissolved in acetic acid (50 μl, 80% v/v) and NCS (2 mg/ml) in dimethylformamide was added to a 10-fold molar excess over the total tryptophan residues. After reaction at room temperature for 40 minutes, excess reagent was destroyed with methionine and the solution diluted with water (5 vol.). The peptides were precipitated by adding TCA to 20% w/v, followed by centrifugation. The precipitate was washed with acetone/ HCl (0.2% w/v) and acetone before being dried *in vacuo*.

Control normal albumin samples contained all the reagents except for NCS.

Peptide analysis is described in Chapter 6.

5.3 RESULTS AND DISCUSSION

Partial digestion of albumins Redhill and Warwick-2 with carboxypeptidase Y revealed that the C-terminal residue of both variants is L-leucine. The mobility of the C-terminal amino acids on t.l.c. (Fig. 5.8) is the same as the mobility of the C-terminal residue of albumin A (L-leucine) and of the standard L-amino acid. In addition, all the albumin samples released glycine which is the penultimate C-terminal residue. No other residues were cleaved under the conditions used. The successful cleavage of the native protein shows that the C-terminal residues are accessible to the enzyme without prior denaturation. The reproducibility of the reaction exemplifies the specificity of the enzyme.

The N-terminal residue of albumin Warwick-2 is L-aspartic acid. This is unchanged from the corresponding residue of albumin A. Fig. 5.9(i) shows the 2-dimensional polyamide chromatograms after separation in three solvent systems. The DNS-asp of albumin Warwick-2 corresponded almost exactly with the standard DNS-asp run on the reverse side of the plate and was distinctly separated from any DNS amino acids which run close to it during

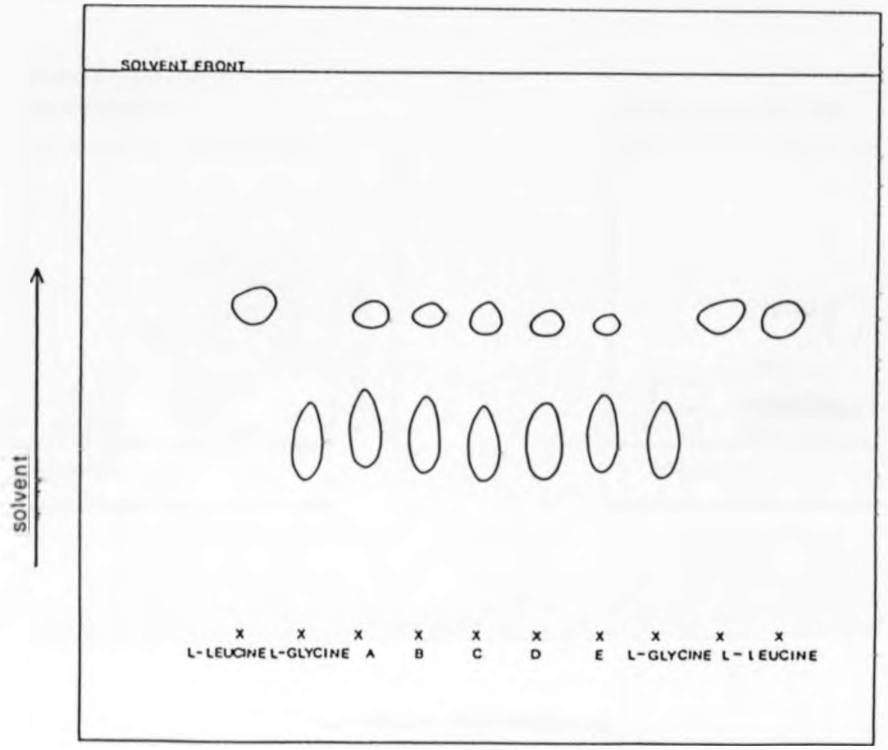

Fig. 5.8 Identification of the C-terminal amino acids of albumins Redhill and Warwick-2 by thin layer chromatography.
A, albumin A/albumin Warwick-2; B, albumin Warwick-2; C, albumin A; D, albumin A/albumin Redhill; E, albumin Redhill.

t.l.c. The third solvent was required to resolve DNS-asp from DNS-OH.

The control experiment using albumin A gave the expected L-aspartic acid as the N-terminal residue.

Fig. 5.9(ii) shows the chromatograms obtained for albumin A/albumin Redhill. After running in the third solvent system, the DNS-asp spot of albumin A was readily visible. However, this solvent is unable

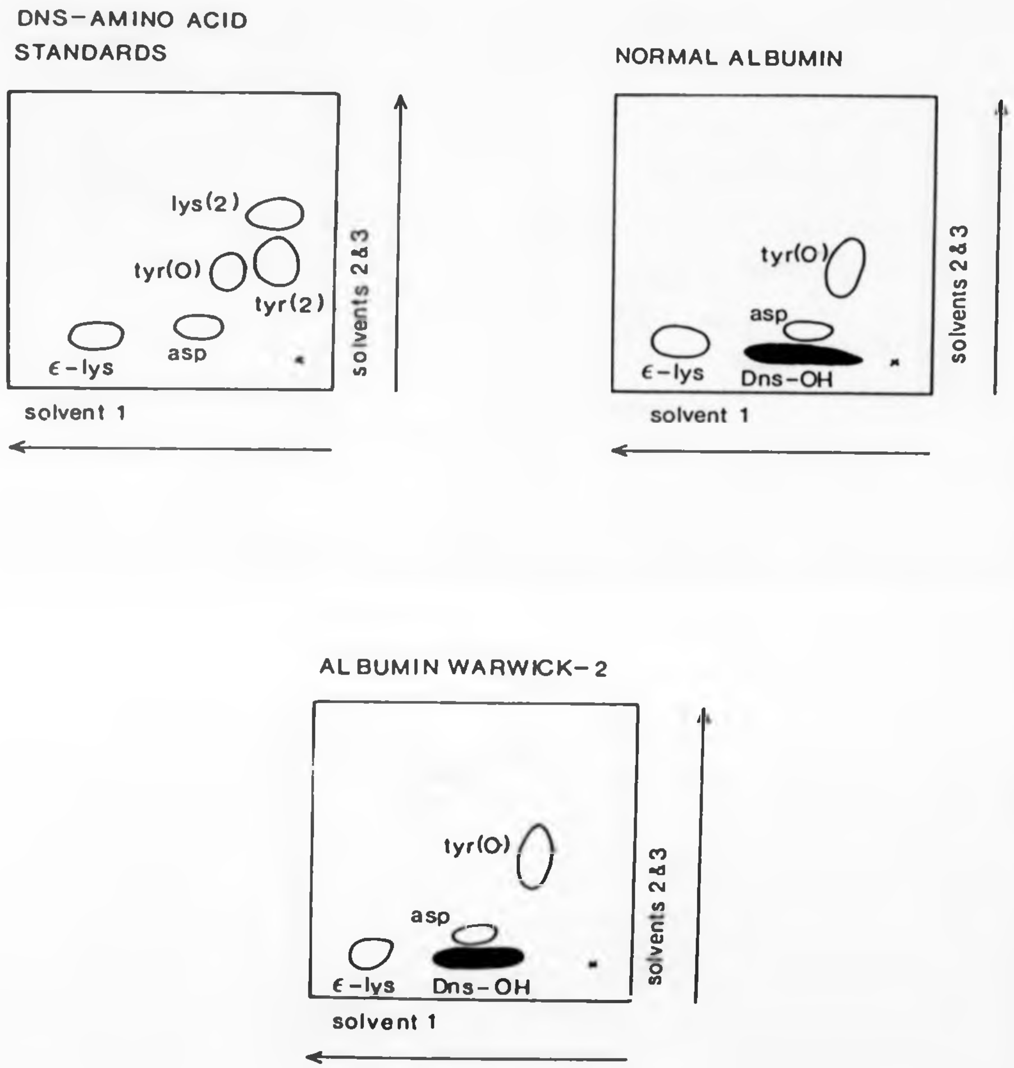

Fig. 5.9(i) Identification of the N-terminal amino acid of albumin Warwick-2.

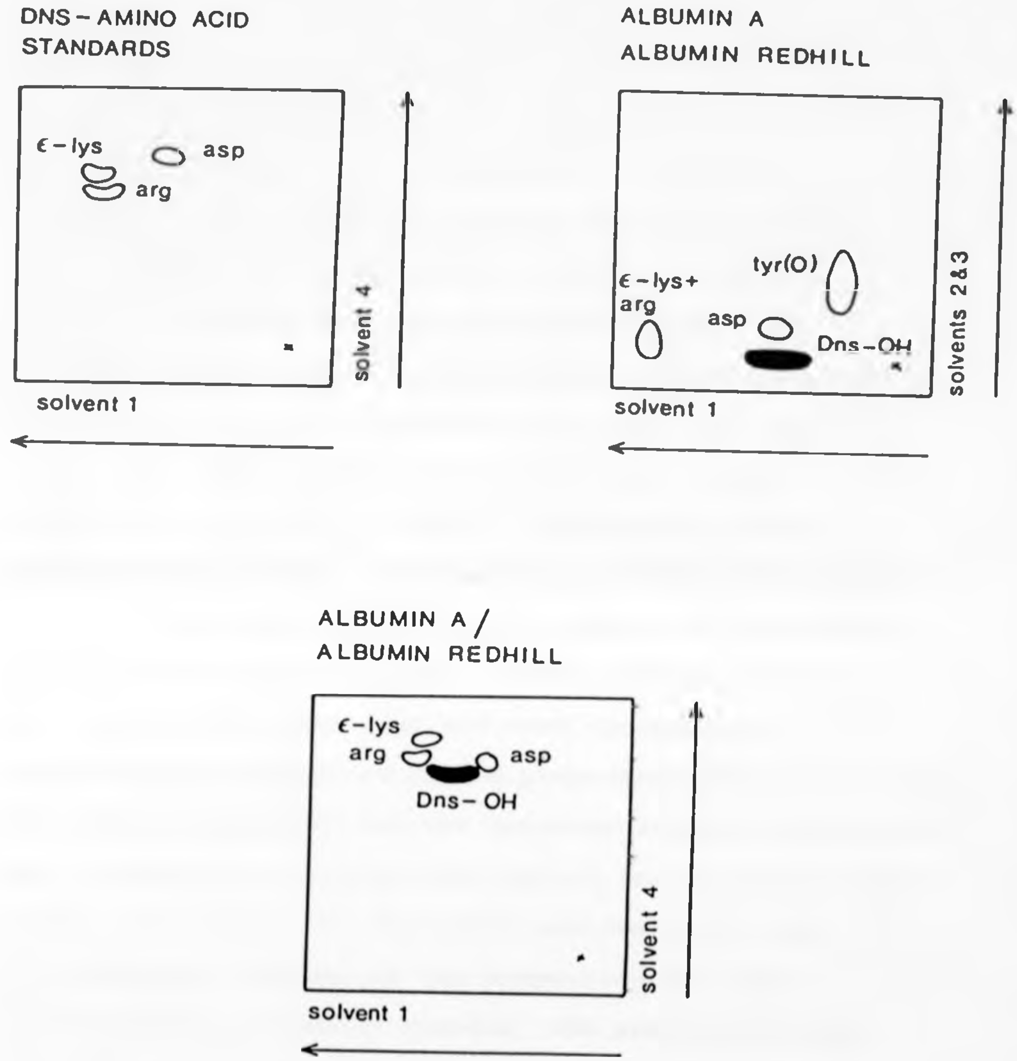

Fig. 5.9(ii) Identification of the N-terminal amino acid of albumin Redhill.

to resolve DNS-ε-lys and DNS-arg. Following chromatography in the fourth solvent, DNS-arg is clearly resolved from DNS-ε-lys showing that the N-terminal amino acid of albumin Redhill is L-arginine. This indicates strongly that albumin Redhill is a proalbumin. The basic propeptide contributes to the slow mobility of albumin Redhill during electrophoresis at pH 8.6. The two dansyl derivatives are also distinguishable on the basis of colour. The fluorescence of DNS-arg is slightly more yellow than DNS-ε-lys which appears slightly blue at 365 nm. These colour differences were readily observed both in the N-terminal derivatives and the standard amino acids. DNS-asp has a yellow fluorescence.

The inconclusive results obtained from partial tryptic digestion of albumin Redhill (Section 3.2.8) can therefore be clarified and some speculation concerning the structure of the propeptide may be permitted. The normal propeptide has the sequence arg-gly-val-phe-arg-arg. Trypsin is specific for arginyl (and lysyl) peptide bonds except where the following residue is proline. If the C-terminal residue of the propeptide has been substituted by a proline residue, the propeptide would not be cleaved except by very prolonged digestion which may also fragment the whole protein, and the electrophoretic mobility of the variant albumin would remain unchanged, as was observed both at pH 5.0 and pH 8.6. Considering the nature of the base changes in the genetic code that would be required to bring about this change, the codons for arginine are CGU, CGC, CGA, CGG, AGA and AGG while the codons for proline are CCU, CCA, CCC or CCG. An arginine to proline substitution requires only one base change and is therefore one of the more likely mutations that might occur. Had the propeptide been cleaved, the loss of a strongly basic segment of the sequence should initiate some change in the electrophoretic mobility, even if the mobility of the variant did not revert completely to that of albumin A. After treatment with trypsin, the variant should migrate more rapidly towards the anode. No such change was observed. As mentioned previously (Section 3.3), metal binding studies support the evidence for a proalbumin.

The combined results obtained from molecular weight studies, tryptic digestion and N-terminal residue determination strongly suggest that albumin Redhill is a proalbumin possessing two sites of mutation, one within the propeptide and the other, possibly involving the insertion of a short sequence elsewhere in the molecule. Albumin Warwick-2 behaves like other monomeric alloalbumins having undergone a single amino acid substitution. Since this variant is also of the slow type, the mutation must also involve some loss of negative charge.

Analysis of the peptides derived from chemical fragmentation of albumins Redhill and Warwick-2 are discussed separately in the following chapter.

CHAPTER 6

PEPTIDE MAPPING

6.1 INTRODUCTION

This chapter details the analytical and preparative methods used to compare and isolate the peptides derived from the chemical cleavage reactions.

CNBr peptides were analysed using the electrophoretic procedure of Franklin *et al.* (1980) and by analytical isoelectric focusing in polyacrylamide gel. Fragments resulting from o-iodosobenzoic acid cleavage were examined using a similar electrophoretic system and also by SDS-PAGE (Swank and Munkres, 1971) and SDS-disc PAGE (Brown *et al.*, 1967), whereas NCS peptides were resolved by SDS-disc PAGE (Laemmli, 1970).

6.1.1 Analysis of CNBr Peptides by PAGE (Franklin *et al.*, 1980)

Peptides were resolved on 12% polyacrylamide gels containing 5 M urea, 5% acetic acid and 6 mM Triton X-100. This system gave highly reproducible peptide maps and is sensitive to small changes in the ionic charge of the peptides. The cationic fragments are differentially retarded depending on their ability to bind the non-ionic detergent and to form micelles between the detergent and the hydrophobic regions of the peptide chain. The resultant mobility, therefore, arises through a combination of the molecular size, net charge and degree of hydrophobicity of the protein (Zwiedler and Cohen, 1972). Unlike other, conventional electrophoretic systems, these gels were able to resolve neutral amino acid substitutions. Improved resolution and clarity of the peptide maps was obtained on gels measuring 47 cm in length as opposed to the usual length of 16 cm.

The peptides were identified using Franklin's notation.

6.1.2 Analysis of CNBr Peptides by Analytical Isoelectric Focusing

Isoelectric focusing has rarely been used for separating peptides since they often have no clearly defined isoelectric points. A further problem is that peptides have very similar properties to the carrier ampholytes in that both are amphoteric and have comparable molecular weights. Therefore, although peptides may focus well, their detection and recovery may be difficult. Small peptides are poorly fixed in TCA and tend to be washed out of the gel along with the ampholytes.

The solubility of peptides in aqueous solution is usually low and various additives are incorporated into the gel to preserve the solubility of focused bands. To this end, 5 M urea was included in the gel and artefacts due to oxidation of methionine residues (cysteine residues were already stabilised by carboxymethylation) were counteracted by including ascorbic acid. This anti-oxidant has an additional advantage over thiodiglycol, 2-mercaptoethanol and dithiothreitol in that it prevents possible modification of tyrosine residues (Jacobs, 1973).

Isoelectric focusing was carried out with a view to separating the CNBr peptides by chromatofocusing. The large peptides presented few problems during IEF. Reproducible band patterns were obtained using conventional focusing procedures in the range pH 3-10.

6.1.3 Preparative Isolation of Peptides

Established procedures for the preparative purification of peptides include gel filtration, ion-exchange chromatography and preparative electrophoresis. Considering the proportionally minute difference between the abnormal peptides and the corresponding peptides from albumin A, and also the difficulty in separating the whole albumins by chromatographic methods, it was not expected that the peptides would be separable by chromatography. The only sufficiently discriminating method was the electrophoretic system of Franklin *et al.* Also, with this system, the identity of the peptide obtained was certain.

The reproducible, well-defined band patterns obtained with analytical IEF (Section 6.2.2) indicated that the CNBr peptides had characteristic, widely distributed, and reproducible isoelectric points.

Chromatofocusing was not expected to be able to isolate individual peptides since the isoelectric points of some of the peptides were very closely spaced. The rationale behind the procedure was to obtain a degree of separation such that the abnormal peptides were separated from some of the normal ones, facilitating the subsequent purification of the abnormal fragments.

6.2 EXPERIMENTAL

6.2.1 Peptide Mapping of CNBr Peptides by PAGE (Zwiedler, 1978; Franklin *et al.*, 1980)

Gels (16 x 16 x 0.15 cm) containing 12% acrylamide, urea (5 M), acetic acid (5%) and Triton X-100 (6 mM) were overlayered with acetic acid (5% v/v) containing urea (5 M) and Triton X-100 (6 mM) and pre-electrophoresed at 130 V constant voltage for 4 hours or until a minimum current had been reached (typically 12 mA). Pre-electrophoresis was continued for a further 30 minutes at 130 V under acetic acid (5% v/v) containing urea (5 M), Triton X-100 (6 mM) and 2-mercaptoethanol (0.5 M). Acetic acid (5%) formed the electrode buffer.

The peptides were dissolved in a suitable volume of urea (8 M)/thiodiglycol (1% v/v)/dithiothreitol (20 mM) and reduced for 90 minutes at 37^{o}C. Basic fuchsin (5 μl, 1% w/v in water) was included as the tracker dye. Electrophoresis was carried out at

130 V for 16 hours with acetic acid (5% v/v) as the electrode buffer.

For better resolution, larger gels (20 x 47 x 0.15 cm) were used. Pre-electrophoresis was at 340 V for 24 hours, followed by electrophoresis of the peptides at 300 V for 48 hours.

6.2.1.1 Detection of the Peptides

Gels were simultaneously fixed and stained in PAGE Blue G-90 (0.2% w/v) in acetic acid (10% v/v)/ methanol (45% v/v) and destained by diffusion in acetic acid (10% v/v)/methanol (45% v/v) at 40°C.

6.2.2 Isoelectric Focusing of CNBr Peptides

Electrofocusing gels (T = 5%, C = 3%) containing Pharmalyte pH 3-10, urea (5 M), methylamine (25 mM, cyanate trap) and L-ascorbic acid (0.1 mM) were cast into tubes (9 x 0.6 cm i.d.) as previously described (Section 3.2.9.1). Persulphate and any cyanate ions were removed by prefocusing at 1 mA/gel for 30 minutes.

CNBr peptides were dissolved in urea (8 M)/thiodiglycol (1% v/v)/dithiothreitol (20 mM) and reduced for 90 minutes at 37°C. An equal volume of a solution containing Pharmalyte pH 3-10 (diluted 1:7.5 in water)/sucrose (30% w/v)/ methyl red was added and approximately 250 μg of peptides was applied per gel. Focusing was at 1-2 mA/gel for 3 hours with cooling. The electrolytes comprised phosphoric acid (1.0 M) at the anode and NaOH (1.0 M) at the cathode. Both solutions contained ascorbic acid (0.1 mM) and were degassed before use.

6.2.2.1 Fixing, Staining and Destaining

(i) With PAGE Blue G-90 (Righetti and Drysdale, 1974)

Following overnight fixing in methanol (30% v/v)/sulphosalicyclic acid (3.5% w/v)/TCA (11.5% w/v), gels were immersed for 4-6 hours in a solution of PAGE Blue G-90 (0.05% w/v) containing copper sulphate (0.1% w/v) in acetic acid (19% v/v)/ethanol (25% v/v). Gels were then destained for 4 hours in the same solution but containing only 0.01% w/v of the dye, and finally in aqueous acetic acid (10%)/ ethanol (10%) at 40^{o}C. The inclusion of copper sulphate in the stain reduces the background colouration.

(ii) With FAST GREEN FCF (Riley and Coleman, 1968)

This less sensitive stain was useful for depicting major bands. Immediately after focusing, gels were fixed in sulphosalicyclic acid (5% w/v)/TCA (16.7% w/v) and then stained for 6 hours in Fast Green FCF (0.25% w/v) dissolved in acetic acid (10% v/v)/ethanol (30% v/v). Destaining was at 40^{o}C in acetic acid (10% v/v)/ ethanol (10% v/v).

6.2.2.2 Measurement of the pH Gradient

Blank gels containing Pharmalyte but no sample were run simultaneously. After focusing, gels were cut into 5 mm lengths and each segment eluted in degassed water (1.0 ml) for several hours in a sealed vial. The pH of each solution was then measured with a pH microelectrode.

6.2.3 Analysis of Tryptophanyl Peptides

6.2.3.1 o-Iodosobenzoic Acid Peptides

(i) By Urea/Acid/Triton X-100 PAGE (Zwiedler, 1978)

Gels (16 x 16 x 0.15 cm) contained 10% acrylamide, urea (5 M), acetic acid (5%) and Triton X-100 (6 mM). Sample preparation and pre-electrophoresis were carried out as described for CNBr peptides (Section 6.2.1). 250 μg of peptides were applied to each sample well and electrophoresis was carried out at 130 V for 16 hours. Gels were stained as for CNBr peptides (Section 6.2.1.1).

(ii) By SDS-*tris*-phosphate PAGE (T = 7.5%, C = 3.3%) (Swank and Munkres, 1971)

Gels (14 x 0.6 cm i.d.) contained phosphoric acid (0.1 M), SDS (0.1% w/v), urea (5 M) and *tris* to pH 6.8

Peptides were dissolved in SDS (1% w/v)/urea (8 M)/2-mercaptoethanol (1% v/v)/phosphoric acid (0.01 M) adjusted to pH 6.8 with *tris*, heated for 10 minutes at 60^{o}C and stored overnight at room temperature prior to electrophoresis. 125 μg of peptides (in bromophenol blue/sucrose (40% w/v)) were applied per gel and electrophoresis was carried out at 4 mA/gel for 12 hours with phosphoric acid (0.1 M)/SDS (0.1% w/v) adjusted to pH 6.8 with *tris* as the electrode buffer.

Staining and Destaining

Gels were fixed and stained in PAGE Blue G-90 (0.25% w/v) in methanol (45% v/v) and acetic acid (10% v/v). Destaining was at 40°C in methanol (5% v/v)/acetic acid (7.5% v/v) (Weber *et al.*, 1972).

(iii) By SDS-disc PAGE (Brown *et al.*, 1967)

Gels (14 x 0.6 cm i.d.) were a modification of Laemmli's gels, containing 5 M urea. The separating gel (T = 7.5%, C = 2.7%) contained *tris*-HCl (0.375 M, pH 8.9), urea (5 M) and SDS (0.1% w/v) and the stacking gel (T = 3%, C = 2.7%) contained *tris*-HCl (0.125 M, pH 6.8), urea (5 M) and SDS (0.1% w/v). The electrode buffer was *tris* (0.05 M)-glycine (0.38 M) containing SDS (0.1%), final pH 8.3. Samples were dissolved in *tris*-HCl buffer (0.04 M, pH 6.8) containing urea (8 M). Electrophoresis was carried out at 1 mA/gel during stacking and then at 3 mA/gel for 5½ hours.

Fixing, staining and destaining was as above (Weber *et al.*, 1972).

6.2.3.2 N-Chlorosuccinimide Peptides by SDS-disc-PAGE (Laemmli, 1970)

The separating gel contained 10% acrylamide (C = 2.7%). Peptides were dissolved in *tris*-HCl (0.0625 M, pH 6.8) containing glycerol (10%), SDS (2% w/v), 2-mercaptoethanol (5% v/v) and bromophenol blue (0.01% w/v), and heated at 100°C for 2 minutes. 200 μg of peptides was applied to each sample well and electrophoresis was carried out at 60 V constant voltage for 16 hours. Commercial molecular weight markers ranging from 12,300 to 78,000 were run alongside the peptides to aid identification.

Staining and Destaining

The peptides were detected with PAGE-Blue G-90 as described for o-iodosobenzoic acid peptides (Section 6.2.3.1(ii).

6.2.4 Preparative Isolation of CNBr Peptides

6.2.4.1 By Acid/Urea/Triton X-100 PAGE

Preparative gels had a similar composition to the analytical gels, and were of dimensions 20 x 47 x 0.3 cm.

Immediately after electrophoresis, the gel was superficially stained (about 1 hour) in PAGE Blue G-90 (0.2% w/v) dissolved in water. Excess stain was removed by washing in water. The peptides were excised from the gel (Gibson and Gracey, 1979) and finely homogenised in an equal volume of formic acid (60% v/v) using a glass homogeniser with a mechanically driven teflon pestle, followed by extrusion through a glass syringe. The peptides were eluted from the gel by magnetic stirring at 4^{o}C for 6 hours. The acrylamide fragments were removed by centrifugation and the supernatant decanted and lyophilised. The gel was washed twice with formic acid (60%) to ensure maximum recovery.

The dye was removed by adding HCl (6 M) until the dry protein had dissolved. n-Octanol (2 vol.) was added with thorough mixing and the two phases separated by centrifugation. The blue organic layer was removed and the aqueous layer extracted twice more in a similar manner. The final acid phase was diluted with an equal volume of water and lyophilised.

6.2.4.2 By Chromatofocusing

Chromatofocusing of CNBr peptides from normal albumin in the pH range 7-4 was carried out as previously described (Section 4.6). A column (1 x 27 cm) of PBE 74 was equilibrated with imidazole-HCl buffer (0.025 M, pH 7.4) containing urea (5 M), ascorbic acid (0.1 mM) and methylamine (25 mM). CNBr fragments (10 mg) were reduced for 90 minutes at 37^{o}C in urea (8 M)/ thiodiglycol (1% v/v)/dithiothreitol (20 mM) before being applied to the column. Elution was at a flow rate of 18.0 ml/hour using Polybuffer 74-HCl (0.0094 mmol/pH unit/ ml, pH 4.0) containing urea, antioxidant and cyanate scavenger as for the starting buffer. The pH of the fractions eluting either side of the protein peaks was measured and the eluate was monitored at 280 nm.

6.3 RESULTS AND DISCUSSION

Specific cleavage at methionine using cyanogen bromide gave highly reproducible peptide maps on urea/acid/Triton X-100 PAGE. Fig. 6.1 shows the fragmentation patterns of albumin A, albumin A/albumin Redhill and albumin A/albumin Warwick-2. The peptides were assigned according to Franklin *et al.* (1980): on the basis of

Fig. 6.1 Peptide analysis of the CNBr fragments of albumin by acid/urea/Triton X-100 PAGE. A, albumin A from serum containing albumin Warwick-2; Band C, Albumin A/albumin Warwick-2; D, F and G, albumin A/albumin Redhill; E, albumin A from serum containing albumin Redhill; H and I, albumin A.

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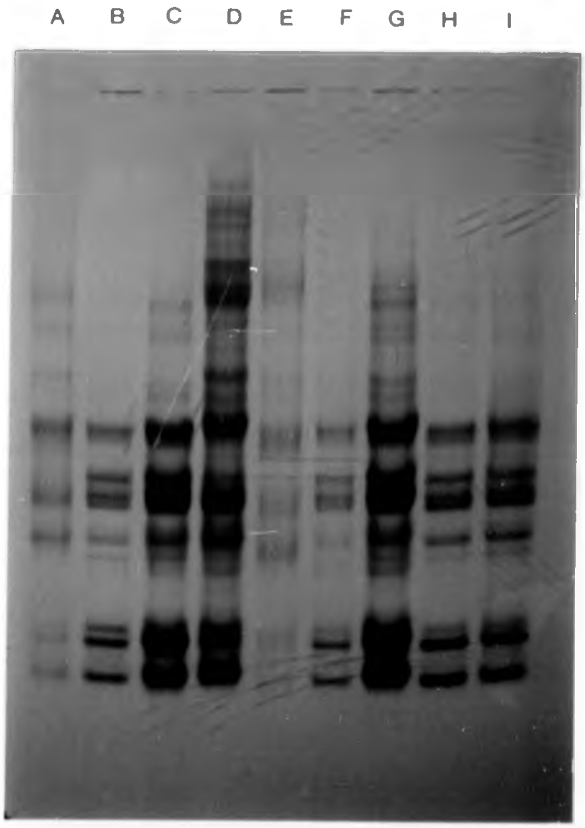

Fig. 6.1 Peptide analysis of the CNBr fragments of albumin by acid/urea/Triton X-100 PAGE. A, albumin A from serum containing albumin Warwick-2; Band C, Albumin Å/albumin Warwick-2; D, F and G, albumin A/albumin Redhill; E, albumin A from serum containing albumin Redhill; H and I, albumin A.

molecular weight and amino acid composition, being numbered in accordance with their order in the known sequence of albumin. Table 6.1 gives the mobilities of the peptides measured relative to the mobility of the basic fuchsin tracker dye.

Table 6.1 Fragments obtained by reaction with CNBr; analysis by acid/urea/Triton X-100 PAGE (Franklin *et al.*, 1980)

Peptide	Residue Number	Relative Mobility (% mobility basic fuchsin)
2 + 3	88-298	0.29
3	124-298	0.33
3 m	124-298	0.35
1	1-87	0.39
1 m	1-87	0.41
1 (albumin Redhill and albumin Warwick-2)	1-87	0.42
5 m	330-446	0.48
5	330-446	0.49
6	447-548	0.53
7	549-585	0.59

Relative mobility is expressed as:

$$\frac{\text{distance migrated by protein}}{\text{distance migrated by tracker dye}}$$

For all albumin samples, fragments 1, 3 and 5 were present as double bands. The minor bands were labelled 'm', referring to peptide modifications probably arising from oxidation of unreacted cysteine or the alkylation of lysine by excess iodoacetic acid during reduction and carboxymethylation. Fragment 7 was present faintly but fragment 4 could not be identified.

For both albumins Redhill and Warwick-2, a faster migrating fragment corresponding to the N-terminal CNBr peptide was present. This band was not visible in the peptide maps of albumin A indicating that both variants possess a site of mutation in the N-terminal region of the molecule, between residues 1 and 87. The faster mobility is coincident with the finding of a basic propeptide in albumin Redhill. The variant peptides are indistinguishable from each other on this gel system and an alteration in the Triton binding conditions may be required to distinguish them.

The similarity between the peptide maps of albumin Redhill and albumin A suggests that the second mutation involving the increased molecular weight of the variant does not interfere with cleavage at methionine residues and may therefore lie within one CNBr fragment and not span a greater number of peptides.

For albumin Warwick-2, the faster migration of the variant peptide at pH 3.0 indicates a loss of negative charge. Consequently, a change from an acidic or neutral residue is suspected, analogous to fragment 7 of albumins B and Mexico-2 (Section 1.8.2.12 and 1.8.2.13). No other novel peptides were detected for either variant on this gel system.

Peptide mapping using the mixed albumins was more informative than studies involving the variants alone since preparations of the pure variants tended to give less well-resolved peptide maps, the probable cause being amino acid modification during the long chromatographic and electrophoretic purification procedures. Also, identification of the novel peptides was simplified if the normal peptide was present as a reference.

Isoelectric focusing of the CNBr peptides exemplified the difference between albumins Redhill and Warwick-2. The band patterns for all samples were closely reproducible and span a wide pH range, from pH 7.35 to pH 4.05. Normal albumin gave eight major, well separated bands which were more evident after staining with Fast Green FCF than with PAGE Blue G-90. The major bands were interspersed with many closely spaced minor bands which stained more intensely with the more sensitive PAGE Blue, obscuring the main bands to some extent and complicating the interpretation of the peptide maps. The pI's were as follows, as measured using the pH gradient curve shown in Fig. 6.2, pH <u>7.35</u>, 7.05, <u>6.85</u>, <u>6.35</u>, 6.25, 6.05, 5.95, 5.9, <u>5.7</u>, <u>5.5</u>, <u>5.2</u>, 5.0, 4.9, 4.8, <u>4.7</u>, 4.55, 4.45, <u>4.05</u>. The major bands are underlined.

Albumin A/albumin Redhill fragments also showed the same eight major bands corresponding to the expected number of CNBr peptides, but no differences between the isoelectric patterns of albumin Redhill and albumin A were apparent. The variant peptides may have been obscured by the multitude of minor bands or become isoelectric outside the pH range employed in this analysis. The

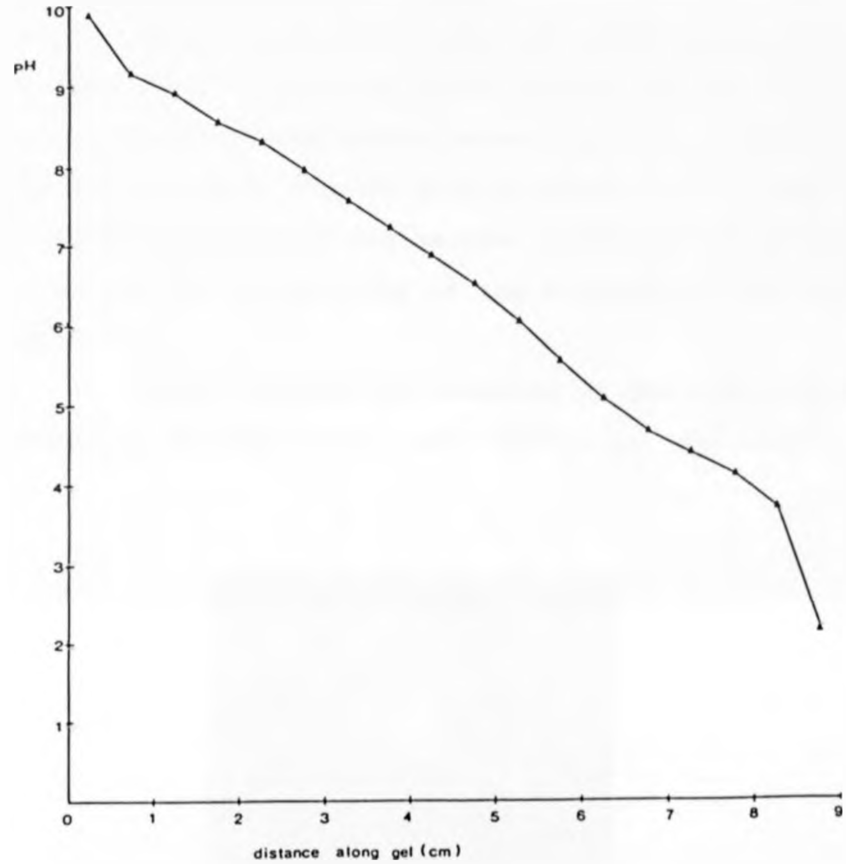

Fig. 6.2 Isoelectric focusing of CNBr peptides in the pH range 3-10: pH gradient curve.

minor bands probably occurred as a consequence of amino acid modifications: oxidation of non-alkylated cysteine residues, deamidation of asparagine and glutamine or carbamylation of amino groups by residual cyanate, or may be due to the natural heterogeneity inherent in albumin preparations.

Albumin Warwick-2 clearly showed an additional acidic peptide with a pI of 4.3.

Fig. 6.3 shows the peptide maps stained with Fast Green FCF. The pI of the abnormal peptide is more basic than the corresponding normal peptide, there being a difference between them of 0.25 pH units. This agrees with the slower mobility of the native variant at pH 8.6 and 5.0 and the faster mobility of the N-terminal CNBr peptide at pH 3.0.

Since isoelectric focusing of the CNBr peptides resulted in so many bands, this method was not very useful

Fig. 6.3 Isoelectric focusing of CNBr peptides in the pH range 3-10, A, albumin A; B and C, albumin A/albumin Redhill; D and E, albumin A/albumin Warwick-2.

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for identifying the abnormal peptides. The only assignable peptide was that derived from the N-terminus and previously identified as containing mutations by acid/urea/Triton X-100 PAGE.

Specific cleavage at tryptophan with o-iodosobenzoic acid and N-chlorosuccinimide gave further information concerning the location of the second mutation in albumin Redhill.

All three gel systems employed to analyse the fragments obtained from o-iodosobenzoic acid cleavage revealed that this reagent cleaved the albumin specifically at tryptophan. However, repeated attempts showed the reaction to be non-reproducible and subsequent fragmentation patterns were variable.

Fig. 6.4 shows the peptide maps obtained by extended electrophoresis on 10% acid/urea/Triton X-100 gels. Control experiments with no included reagent showed that no anomalous peptides occurred as a result of random, non-specific hydrolysis in the solvent (Brown *et al.*, 1967). This gel system was unable to resolve the abnormal peptides from their normal analogues. The tryptophanyl peptides are much larger than the CNBr peptides separated in the same system, and a contributory factor to the lack of resolution may be that the mutation is proportionally much smaller relative to these larger peptides. Albumin A, albumin A/albumin Redhill and albumin A/albumin Warwick-2 gave identical fragmentation patterns comprising three bands which corresponded to the uncleaved albumin, the C-terminal peptide and the

Table 6.2 Fragments obtained by reaction with o-iodosobenzoic acid; analysis by acid/urea/ Triton X-100 PAGE (Franklin *et al.*, 1980)

Sample	Relative Mobility (% mobility basic fuchsin)	Identity of Protein Band
Albumin A (control)	0.08	Uncleaved albumin
Albumin A	0.08	Uncleaved albumin
+ o-iodosobenzoic acid	0.16	C-terminal peptide (residues 215-585)
	0.28	N-terminal peptide (residues 1-214)
Albumin A/albumin Redhill	0.08	Uncleaved albumin
+ o-iodosobenzoic acid	0.16	C-terminal peptides
	0.28	N-terminal peptides
Albumin A/albumin Warwick-2	0.08	Uncleaved albumin
o-iodosobenzoic acid	0.17	C-terminal peptides
	0.28	N-terminal peptides

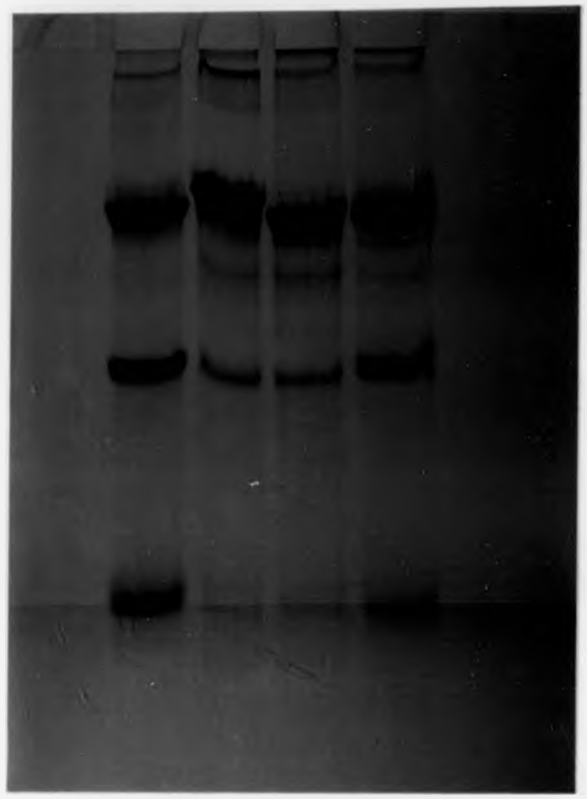

Fig. 6.4 Analysis of o-iodosobenzoic acid peptides by acid/urea/Triton X-100 PAGE (Franklin *et al.*, 1980). A, pure albumin A; B, albumin A; C, albumin A/albumin Warwick-2; D, albumin A/albumin Redhill.

lower molecular weight N-terminal peptide (Table 6.2). It was necessary to overload each gel in order to see the peptides clearly since cleavage yields were quite low.

The SDS-*tris*-phosphate gels (Swank and Munkres, 1971) and SDS-disc gels (Brown *et al.*, 1967) were able to resolve the variant C-terminal peptide of albumin Redhill on the basis of molecular weight. Fig. 6.5 shows the band patterns obtained using SDS-*tris*-phosphate

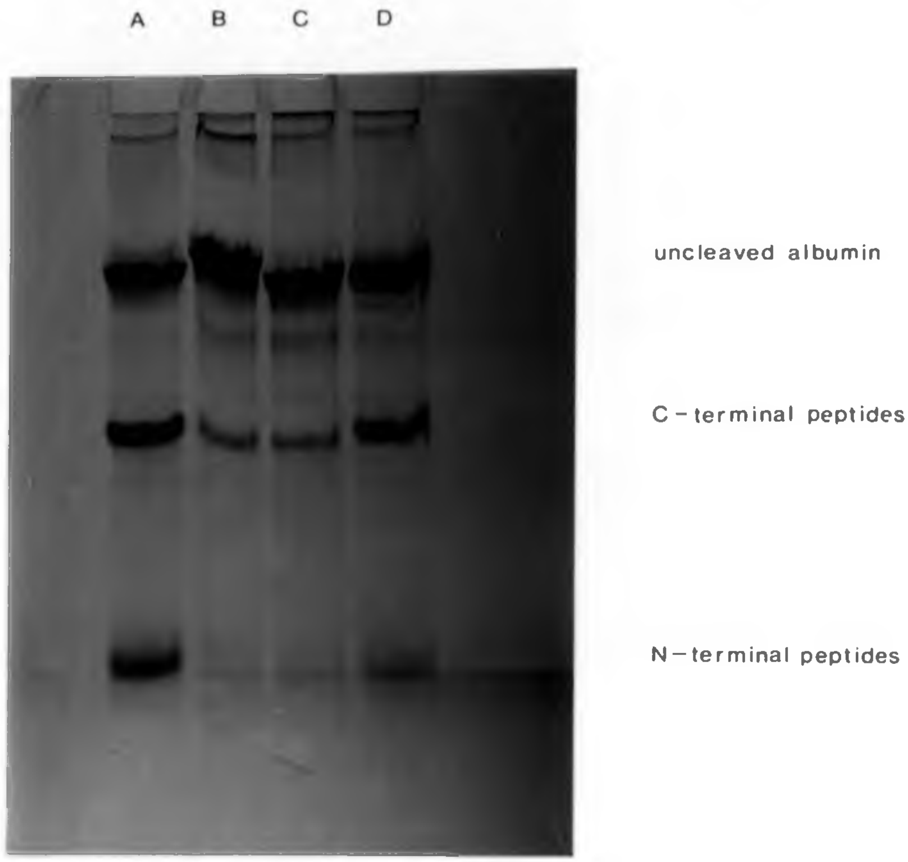

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Fig. 6.5 Analysis of o-iodosobenzoic acid peptides by SDS-*tris*-phosphate PAGE (Swank and Munkres, 1971). A, standard molecular weight markers; B, uncleaved albumin A (control); C, albumin A; D, albumin A/albumin Redhill; E, albumin A/albumin Warwick-2.

gels. The molecular weights of the peptides bear no resemblance to the molecular weights of the standard markers. The C-terminal peptide of albumin Redhill has a higher molecular weight than the corresponding peptide of albumin A. The two peptides are well-resolved, being visible as distinct bands of equal intensity on both electrophoretic systems. The increased molecular weight previously observed during conventional molecular weight determination (Section 3.2.7) therefore lies in the

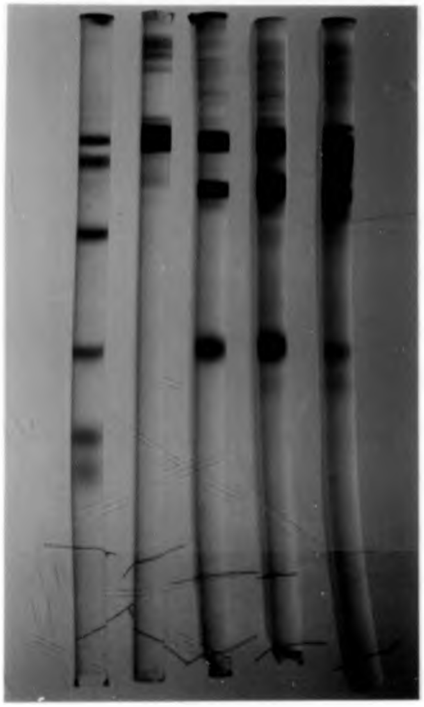

Fig. 6.5 Analysis of o-iodosobenzoic acid peptides by SDS-*tris*-phosphate PAGE (Swank and Munkres, 1971). A, standard molecular weight markers; B, uncleaved albumin A (control); C, albumin A; D, albumin A/albumin Redhill; E, albumin A/albumin Warwick-2.

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C-terminal region of the variant albumin, between residues 215 and 585. Thus, albumin Redhill has two separate regions of mutation. The abnormal peptide in albumin Warwick-2 was indistinguishable on all the gel systems employed, indicating a smaller degree of mutation, too minute to cause a detectable change in molecular size, reminiscent of a single amino acid substitution.

The faint band visible below the N-terminal peptide in the peptide maps of both variants on the SDS-*tris*-phosphate system was not present on the SDS-disc-PAGE system. The intensity of this band is much lower than the intensity of the main band and may represent a low molecular weight fragment arising from a small degree of cleavage at tyrosine residues. It is unlikely that the mutation occurring in the N-terminal region of albumin Warwick-2 involves a reduction in size; albumin Redhill has an increased molecular size in this region and shows the same faint band. Furthermore, mutations involving a deletion of part of the sequence have not been discovered in any other variant studied in this depth, and no low molecular weight bands were observed during studies with the native proteins. Alternatively, the additional band may therefore be an artefact arising from an altered binding capacity of the variant albumins for SDS. The primary structure of the protein influences its ability to bind SDS, possibly involving electrostatic attractions. The N-terminal peptides of both variants possess additional positive charge. Electrostatic attraction may cause more of the anionic

Table 6.3 Fragments obtained by reaction with o-iodosobenzoic acid; analysis by SDS-PAGE

Sample	Relative mobility (% mobility bromophenol blue) Swank and Munkres (1971)	Relative mobility (% mobility bromophenol blue) Brown *et al.* (1967)	Identity of protein band
Standard molecular weight markers	0.22	0.36	Ovotransferrin
	0.25	0.42	BSA
	0.39	0.72	Ovalbumin
	0.59	0.94	Chymotrypsinogen A
	0.74	0.98	Myoglobin
	0.80	-	Cytochrome C
Albumin A (control)	0.21	0.28	Uncleaved albumin
Albumin A + o-iodosobenzoic acid	0.21	0.28	Uncleaved albumin
	0.29	0.43	C-terminal peptide (residues 215-585)
	0.55	0.78	N-terminal peptide (residues 1-214)
Albumin A/ albumin Redhill + o-iodosobenzoic acid	0.20	0.29	Uncleaved albumin
	0.26	0.42	C-terminal peptide (albumin Redhill)
	0.29	0.44	C-terminal peptide (albumin A)
	0.53	0.78	N-terminal peptide (albumin A/albumin Redhill)
Albumin A/ Albumin Warwick-2 + o-iodosobenzoic acid	0.21	0.28	Uncleaved albumin
	0.29	0.43	C-terminal peptide (albumin A/albumin Warwick-2)
	0.55	0.77	N-terminal peptide (albumin A/albumin Warwick-2)

SDS to bind to this region, thus increasing the overall negative charge on the peptide. During electrophoresis, the variant peptides migrate more rapidly to the anode giving a false impression of low molecular weight bands. Table 6.3 gives the relative mobilities of the tryptophanyl peptides in both the Swank and Munkres and Brown gel systems. There is no comparison between the mobilities of the peptides in the two systems but the fragmentation patterns give the same information.

Fig. 6.6 shows the fragmentation patterns obtained after reaction with N-chlorosuccinimide. This reagent proved to be more reliable than o-iodosobenzoic acid and subsequent reactions gave similar peptide maps. But, like o-iodosobenzoic acid, yields were rather low and it was necessary to overload the gels in order to see the peptides clearly.

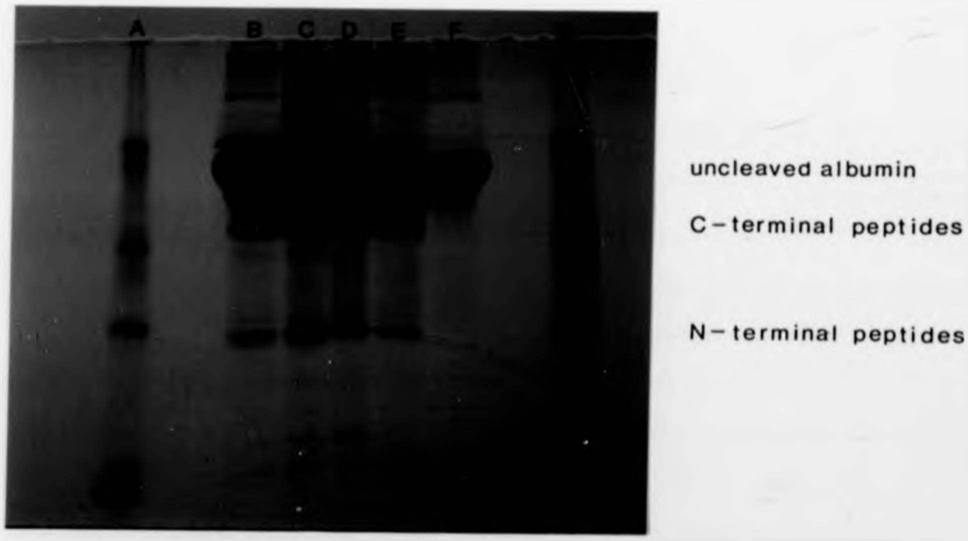

Fig. 6.6 Analysis of N-chlorosuccinimide peptides by SDS-PAGE (Laemmli, 1970).
A and G, standard molecular weight markers; B, albumin A/albumin Warwick-2; C, albumin A/albumin Redhill; D & E, albumin A; F, uncleaved albumin A (control).

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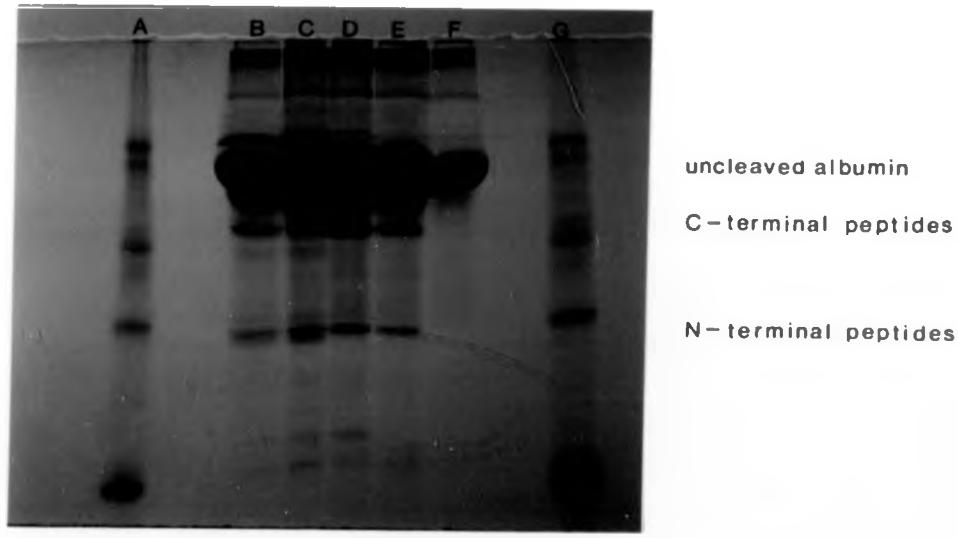

Fig. 6.6 Analysis of N-chlorosuccinimide peptides by SDS-PAGE (Laemmli, 1970).
A and G, standard molecular weight markers; B, albumin A/albumin Warwick-2; C, albumin A/albumin Redhill; D & E, albumin A; F, uncleaved albumin A (control).

Table 6.4 Fragments obtained by reaction with N-chlorosuccinimide; analysis by SDS-PAGE (Laemmli, 1970)

Sample	Relative mobility (% mobility bromo-phenol blue)	Identity of protein band	Molecular weight
Standard molecular weight markers	0.21	Ovotransferrin	76-78,000
	0.24	BSA	66,200
	0.41	Ovalbumin	45,000
	0.57	Chymotrypsinogen A	25,700
	0.86	Myoglobin	17,200
	0.90	Cytochrome C	12,300
Albumin A (control)	0.27	Uncleaved albumin	
Albumin A + NCS	0.27	Uncleaved albumin	
	0.39	C-terminal peptide (residues 215-585)	47,000
	0.59	N-terminal peptide (residues 1-214)	28,800
Albumin A/ albumin Redhill + NCS	0.26	Uncleaved albumin	
	0.36	C-terminal peptide (albumin Redhill)	49,900
	0.39	C-terminal peptide (albumin A)	47,000
	0.59	N-terminal peptide	28,800
	0.60	N-terminal peptide (albumin Redhill?)	27,700
Albumin A/ albumin Warwick-2 + NCS	0.26	Uncleaved albumin	
	0.39	C-terminal peptide (albumin A/Warwick-2)	47,000
	0.59	N-terminal peptide	28,800
	0.60	N-terminal peptide (albumin Warwick-2?)	27,700

In the Laemmli gel system, the molecular weight of the albumin bands correlated well with the standard markers. Table 6.4 shows the relative mobilities, molecular weights and identification of the peptides. The molecular weight of the C-terminal peptides of albumin Redhill and albumin A differed by a factor of 2,900 in this system. The discrepancy between these measurements and the weight calculated for the native protein (a difference of 2,200) may be a manifestation of the proportional size of the mutation relative to the size of the protein chain in which it is contained or be due to the different mobilities on different concentrations of acrylamide gel. The bands representing the N-terminal peptide in both variants are double and of near equal intensity. The most probable explanation for the duality of these bands lies in the binding of SDS as previously described, especially since they run so close together.

The difference in molecular weight between the C-terminal peptides of albumin Redhill and albumin A and between the whole, native albumin is much larger and the equal intensity of the bands is constant throughout the various gel systems, unlike the varying intensities observed with the N-terminal peptide bands. This strongly suggests that the slower migrating band is not artefactual and represents a true difference in the size of the proteins in this region.

Cleavage with NCS confirms that albumin Redhill has a mutation in both the N- and C-terminal regions, whereas albumin Warwick-2 has one N-terminal mutation.

Specific cleavage of normal and variant albumins at methionine and tryptophan followed by several methods of peptide analysis has shown that albumin Redhill and albumin Warwick-2 are structurally different, both to albumin A and to each other. Albumin Warwick-2 has undergone an amino acid substitution within the first 87 residues from the N-termianl end. Albumin Redhill is a proalbumin with an additional mutation between residues 215 and 585 involving an insertion of about ten to twelve residues.

Preparative isolation of the CNBr peptides from acid/urea/Triton X-100 gels was less successful than originally hoped from the success of the analytical gels. Much of the resolving power and clarity was lost on the larger preparative scale. The normal fragments separated well enough to be excised as separate bands whereas the variant N-terminal peptides tended to merge with the normal peptides owing to the diffuse nature of the bands. Difficulty in obtaining clear backgrounds with diffusion destaining in water reduced the visibility of the abnormal bands and prolonged destaining increased the possibility of losing some of the non-fixed peptides by free diffusion into the destaining solution. Prior fixing in TCA was not favoured since prolonged immersion in strong acid may catalyse the deamidation or some hydrolysis of asparagine and glutamine residues, resulting in the formation of their corresponding acids. This may cause errors in sequencing later on.

Chromatofocusing of CNBr peptides from albumin A resolved the peptides into two peaks. The first group eluted within the pH range 7.59 to 7.39 and the second group between pH 5.88 and pH 3.90. Analysis of these peaks and further use of this technique was hindered by there being no satisfactory method for removing Polybuffer and some difficulty experienced in dialysing out the large quantities of urea employed to maintain the solubility of the peptides during chromatography, owing to the danger of losing small peptides through the dialysis membrane. The molecular weight of some of the smaller peptides is too close to that of Polybuffer to enable separation by gel filtration; the minimum weight that can be separated from Polybuffer by column chromatography is 25,000 and all of the albumin CNBr peptides are smaller than this. Similar difficulties were foreseen with ammonium sulphate precipitation and hydrophobic interaction chromatography, the other suggested methods. The peptides are probably too small to be precipitated with ammonium sulphate and removing the large quantities of this salt required in both methods would meet with the same problems involved in separating the protein from urea.

A more quantitative method for the preparative isolation of the CNBr peptides is required and further investigations are at present being carried out by Mr. P. Matejtschuk in the Department of Chemistry and Molecular Sciences at the University of Warwick, using HPLC as a possible solution to this problem.

Further to the fragmentation studies, samples of purified albumin A and albumin Redhill have been sent to the S.E.R.C.-funded protein sequencing unit at the University of Leeds, where amino acid sequencing studies are at present in progress.

CHAPTER 7

LIGAND BINDING STUDIES

7.1 INTRODUCTION

The relationship between amino acid sequence and ligand binding properties was investigated using nickel, copper, palmitate and bilirubin. The binding regions for these ligands are well established (Section 1.7). The relative binding capacity of albumins Redhill and Warwick-2 compared to the capacity of albumin A provided additional information concerning the site of amino acid substitution.

7.1.1 Nickel and Palmitate

The binding capacity of the variant albumins for the radiolabelled ligands ^{63}nickel and [^{14}C]-palmitate relative to that of albumin A was assessed by cellulose acetate electrophoresis-autoradiography (Brennan and Carrell, 1980).

7.1.2 Copper

Copper binding was measured using gel filtration (Hummel and Dreyer, 1962). The method is based on the following general principle. The protein is dissolved in a solution of a ligand to which it binds. The concentration of free ligand is thereby reduced by an amount equivalent to the protein-ligand complex formed. The solution is applied to a Sephadex column equilibrated with the same solution of ligand as was used to dissolve the protein, and thereafter, eluted with this solution. The concentration of ligand in the eluate is measured. As the protein moves down the column it continues to remove ligand from the solution and elutes in the void volume. The amount of ligand localised in the complex, therefore, rises above that present in the eluant and correspondingly, at some point after the protein peak, the concentration of ligand in the eluate decreases, forming a trough in the elution profile which extends to the salt volume of the column. The quantity of free ligand removed from the solution is equal to the excess concentration of ligand associated with the protein.

7.1.3 Bilirubin

The binding of bilirubin was examined by fluorescence quench titration (Levine, 1977). Albumin, like most proteins exhibits an intrinsic fluorescence due to absorption in the ultraviolet of the aromatic amino acids tryptophan and tyrosine. Tryptophan contributes a greater part of the fluorescence owing to its higher extinction coefficient whereas the fluorescence contribution of phenylalanine is negligible. Bilirubin absorbs strongly in the region of albumin emission so that binding of bilirubin is accompanied by a loss in intrinsic albumin fluorescence due to energy transfer from the excited state tryptophan to the bound ligand (Chignell, 1972; Chen, 1973). To prevent the obscuring of binding sites any endogenous metal ions were removed from albumin by treatment with Chelex 100 before nickel and copper binding. Defatted albumin was used to study palmitate and bilirubin binding.

7.2 EXPERIMENTAL

7.2.1 Removal of Trace Metals from Serum

Small columns (6 x 0.9 cm i.d.) of de-fined Chelex 100 (Na^+ form) were neutralised with four bed volumes of sodium acetate buffer (0.5 M, pH 6.5) followed by equilibration with five bed volumes of water. Sera (100 μl) were applied to individual columns and eluted with water at a slow flow rate. The protein peaks were collected directly into round bottomed flasks (25 ml) and lyophilised.

7.2.2 Nickel Binding (Brennan and Carrell, 1980)

The lyophilised sera were reconstituted with water (100 μl). [^{63}Ni]-nickel chloride (1 mCi) in HCl (0.2 ml, 0.1 M) was diluted with water (0.8 ml) and an aliquot (1 μl, 1 μCi) was added to serum (30 μl). Following incubation at 37^{o}C for 1 hour, the albumins were separated by cellulose acetate electrophoresis in barbitone buffer (0.075 M, pH 8.6) at 8 V/cm for 4 hours. The electrophoretogram was dried in warm air and subjected to autoradiography using LKB Ultrofilm ^{3}H under slight pressure at room temperature for 24 hours. The film was then developed in commercial developer and fixer (5 minutes each solution).

To facilitate interpretation of the autoradiograph, the electrophoretogram was subsequently stained with Ponceau S for comparison.

The control was normal serum with no radiolabelled nickel.

7.2.3 Palmitate Binding (Brennan and Carrell, 1980)

1-[^{14}C]-Palmitate was supplied as a solution in toluene (500 µl, 50 µCi). The lyophilised, defatted normal and mixed albumins (4.2 mg) were dissolved in water (100 µl) and undiluted 1-[^{14}C]-palmitate was added (1 µl, 0.1 µCi per 30 µl of albumin solution). Incubation, electrophoresis and autoradiography followed as previously described. The control was normal albumin with no added radiolabelled ligand.

7.2.4 Copper Binding (Hummel and Dreyer, 1962)

Defatted normal and mixed albumins (5.0 mg), free of trace metals by pre-treatment with Chelex 100, were dissolved in *tris*-HCl buffer (500 µl, 0.02 M, pH 8.0) containing $Cu^{(II)}$ ions (3 µg/ml) and applied to a column (1.6 x 46.5 cm) of Sephadex G-50 pre-equilibrated with the same copper containing buffer. The column was eluted with this buffer at a flow rate of 17 ml/hr and small fractions of equal volume (1.5 ml) were collected. The eluate was primarily monitored at 280 nm. The copper content of the individual fractions was subsequently determined by atomic absorption spectroscopy using a Varian Techtron Spectrophotometer. All measurements were made at 325.1 nm employing the same instrument conditions: Lamp current 3 mA, fuel 3.0, oxidant 7.5 and band width 0.2 nm.

7.2.5 Bilirubin Binding (Levine, 1977)

The titration involved adding aliquots of bilirubin to the albumin solution and measuring the decrease in protein fluorescence after each addition.

Bilirubin (20.8 μM) was initially solubilised in a minimum volume of NaOH (0.1 M) containing EDTA (0.1 mM) and subsequently buffered with *tris*-HCl (0.1 M, pH 7.4). Aliquots (10 μl) of this solution were added to defatted albumin (1.04 μM in *tris* buffer, 2.0 ml) in a 3 ml quartz fluorescence cuvette with a path length of 1 cm, and mixed by inversion five times. A similar cuvette containing buffer only served as the blank and was checked frequently.

The excitation filter had a maximal transmission at 294 nm while the emission maximum was 343 nm. The half band width for both filters was 10 nm. All studies were performed at ambient temperature in subdued lighting and glassware was siliconised with dimethyldichlorosilane solution (2% in 1,1,1-trichloroethane) to prevent adsorption of bilirubin. The shutter allowing light to strike the test solution was opened only when reading fluorescence to avoid photodecomposition of bilirubin.

7.3 RESULTS AND DISCUSSION

The results obtained from the binding of copper and nickel substantiate previous evidence that albumin Redhill is a proalbumin. The mutation within the N-terminal sequence lies within the primary binding site for these two metals, namely the N-terminal tripeptide, whereas the single site of mutation within albumin Warwick-2 lies elsewhere between residues 1 and 87.

Comparing the autoradiograph of serum containing albumin Redhill incubated with ^{63}Ni, and the corresponding cellulose acetate electrophoretogram, the autoradiograph shows only the faster migrating albumin A band. Albumin Redhill failed to give a band on the film. This shows that albumin Redhill is unable to complex nickel in the normal manner, or binds so little that any residual capacity is undetectable by this method. Fig. 7.1 shows the autoradiographs for all sera and the cellulose acetate electrophoretogram stained with Ponceau S is included in Fig. 7.2 for comparison. Control normal serum showed no background radioactivity and there was no detectable nickel uptake by the globulins.

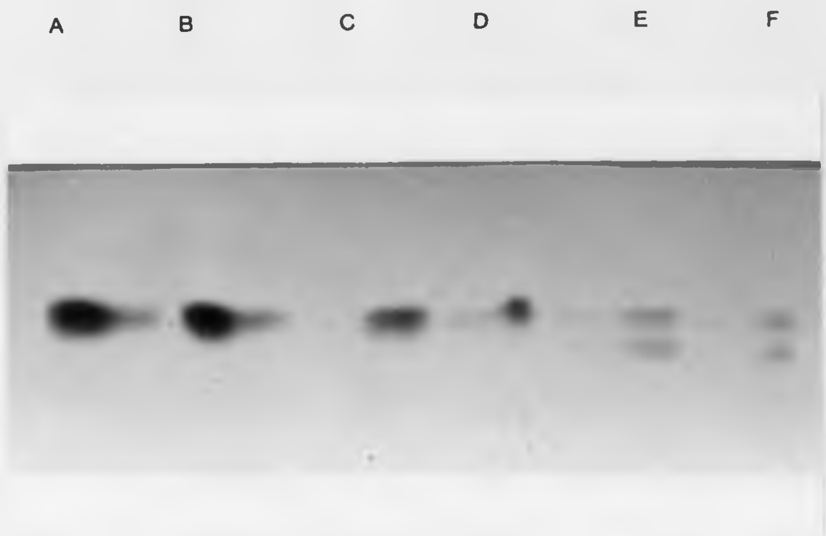

Fig. 7.1 Nickel binding: autoradiograph. E and F, albumin Warwick-2; C and D albumin Redhill; A and B, normal serum.

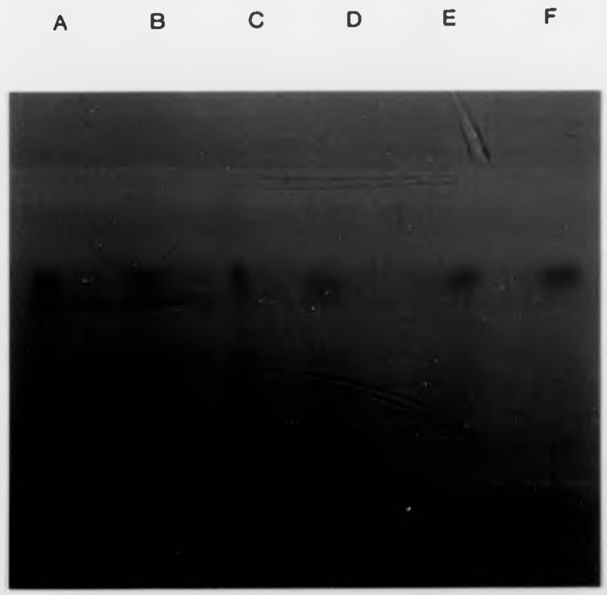

Fig. 7.2 Nickel binding: cellulose acetate electrophoretogram. A and B, albumin Warwick-2; C and D, albumin Redhill; E and F, normal serum.

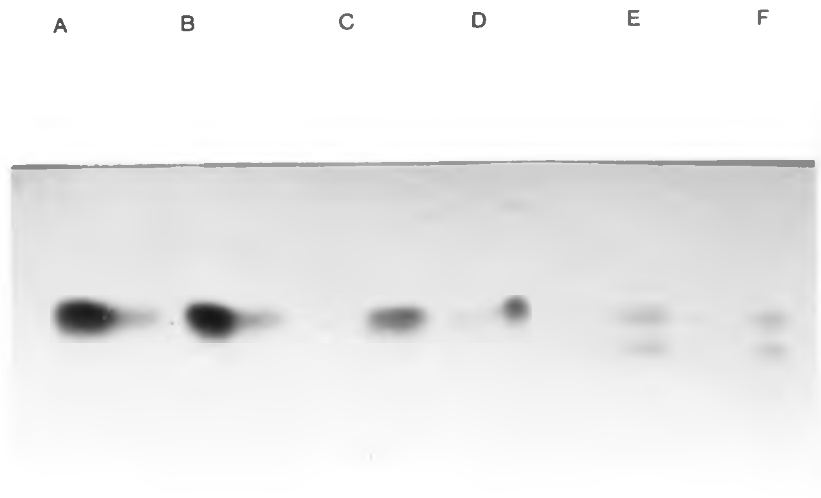

Fig. 7.1 Nickel binding: autoradiograph. E and F, albumin Warwick-2; C and D albumin Redhill; A and B, normal serum.

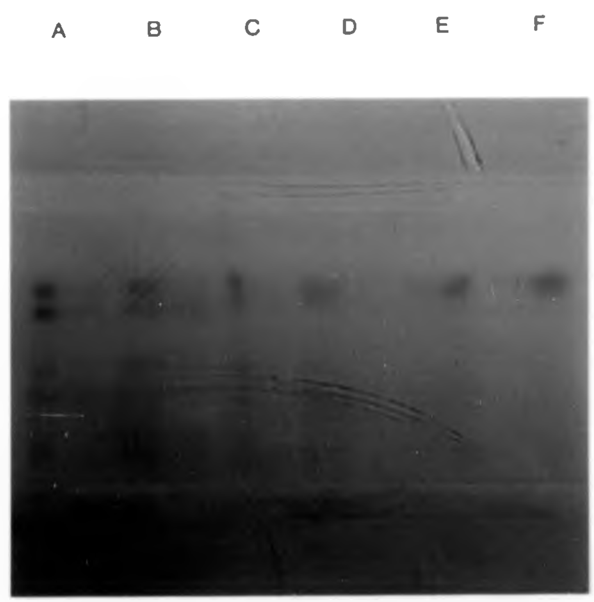

Fig. 7.2 Nickel binding: cellulose acetate electrophoretogram. A and B, albumin Warwick-2; C and D, albumin Redhill; E and F, normal serum.

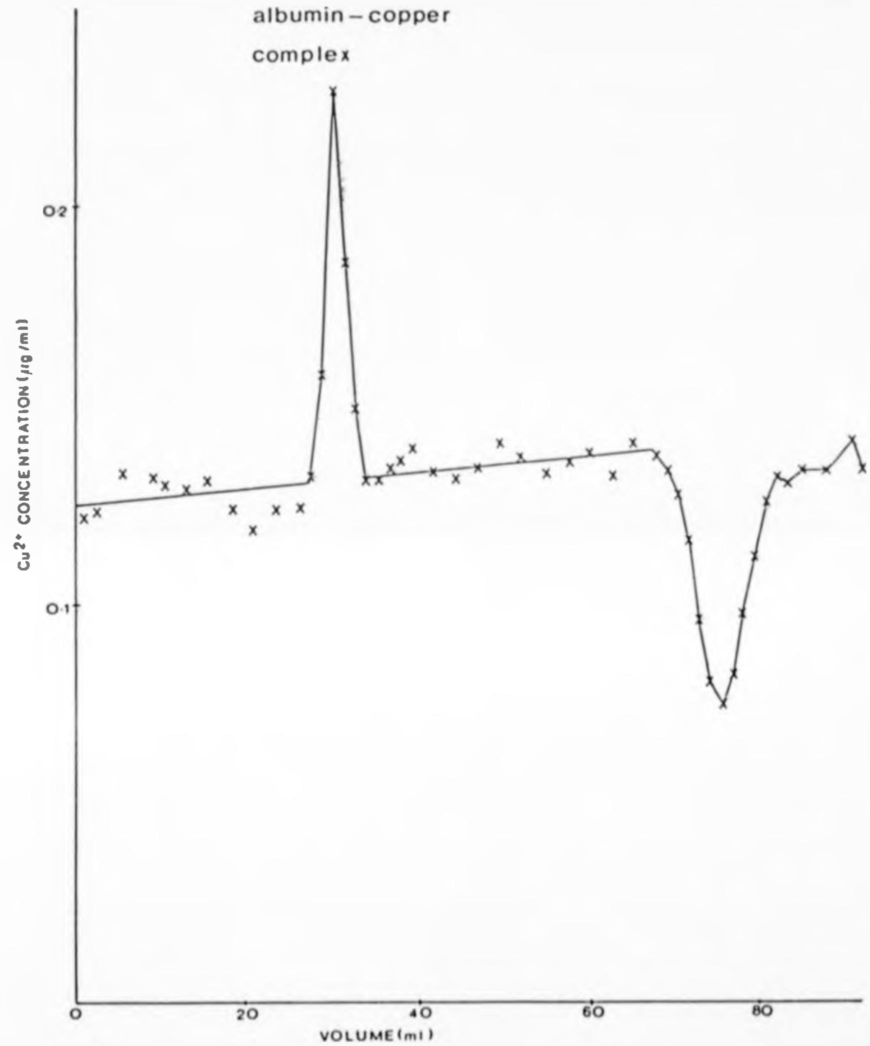

Fig. 7.3 Copper binding: elution profile of albumin Warwick-2 by atomic absorption spectroscopy.

As previously described (Section 1.7.4.3), nickel$^{(II)}$ binds to albumin at one primary site constituting the N-terminal tripeptide NH_2-asp-ala-his. The binding of nickel to albumin variants has been demonstrated as being a test for proalbumins (Brennan and Carrell, 1980). Proalbumin Christchurch showed a similar lack of affinity for nickel. The reduced capacity arises from the unavailability of the α-amino group necessary to complete the chelate structure, which has been blocked by the C-terminal residue of the propeptide. The reduced affinity for copper and nickel is strong evidence for the presence of an N-terminal propeptide in albumin Redhill.

Conversely, both albumin bands in the serum containing albumin Warwick-2 were visible on the film. A densitometric tracing of both bands revealed that the relative proportions of nickel bound by each albumin corresponded to the relative proportion of each albumin in the serum, showing that albumin Warwick-2 binds nickel normally and that the mutation lies further into the sequence between residues 1 and 87. The propeptide of this variant is cleaved normally *in vivo*.

The proposed regions of mutation were confirmed by the copper binding experiments. Fig. 7.3 shows a typical elution profile (albumin Warwick-2) derived from the copper content of the eluate as measured by atomic absorption spectroscopy. The first peak corresponds to the albumin-copper complex eluting in the void volume (27.0 ml as determined with Blue Dextran). The trough represents the copper depleted eluate approximating to the salt volume of the column. The relative areas under the albumin-copper peaks were calculated by taking a standard area under the baseline equivalent to a standard weight of copper. The peak areas for albumin A and albumin A/albumin Warwick-2 were equal and represented 5.48 μg of copper bound per 5.0 mg of the defatted protein, whereas an equal weight of albumin A/albumin Redhill bound only 3.92 μg of copper.

As described previously (Section 1.7.4.2) the primary copper$^{(II)}$ binding site lies within the N-terminal tripeptide and the quantity of copper$^{(II)}$ bound by the variant albumins relative to albumin A shows that albumin Warwick-2 binds this ligand normally at this site whereas albumin Redhill has a markedly reduced capacity. However, this variant does show some affinity for copper, possessing approximately 28% of the binding affinity of albumin A. This copper must be bound at the secondary sites which are unaffected by the alteration in the amino acid sequence.

The abnormality in the C-terminal region of albumin Redhill was investigated using palmitate. The primary binding site for this ligand lies between residues 377 and 503 (Section 1.7.2.5). Autoradiography showed that both variants bound palmitate normally. This was expected for albumin Warwick-2 since no evidence for a mutation in the C-terminal region of the molecule had been found. The C-terminal mutation evident in albumin Redhill did not affect the binding of palmitate and hence it is unlikely that the mutation is located in this region.

Fig. 7.4 shows the fluorescence titration curve for bilirubin binding. The initial, steepest part of each curve represents the binding of one mole of bilirubin per mole of albumin at the primary binding site (residues 186-238, Section 1.7.3.2). The curves for albumin A, albumin A/albumin Redhill and albumin A/ albumin Warwick-2 all coincide in this region indicating

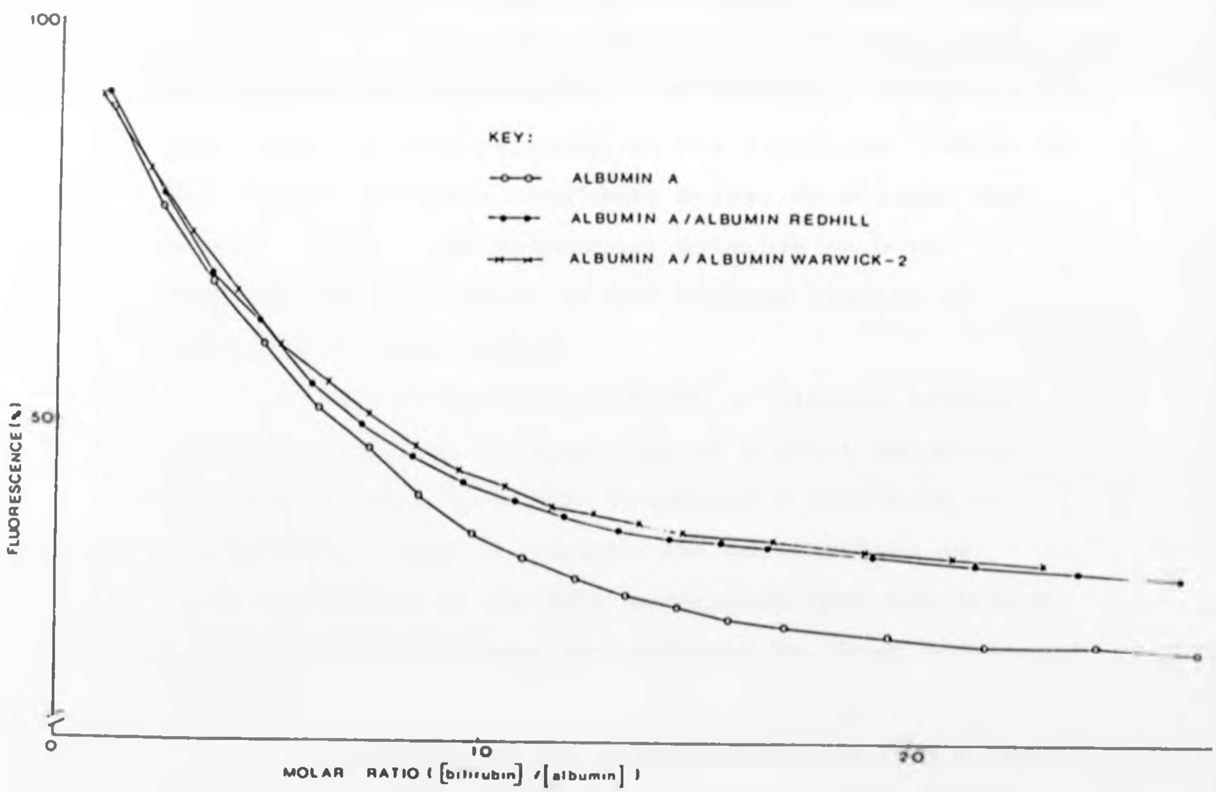

Fig. 7.4 Bilirubin binding: fluorescence titration curve.

that the sequence of both variants is unchanged within the primary bilirubin binding site. In contrast, the curves for both variants depart from the albumin A curve where bilirubin begins to bind at the secondary sites, showing that the mutation in both variants, although different, affects the binding of this ligand at these low affinity sites. Gitzelmann-Cumarasamy *et al.* (1976) localised one of the secondary sites to residues 446 to 547 and the C-terminal mutation in albumin Redhill may lie within this sequence. However, since palmitate (377-503) binding is not affected, and no binding studies involving residues 547 to 585 were carried out, residues 503 to 585 may contain the abnormality. In addition, bilirubin has been shown to bind strongly to the N-terminal region of the albumin molecule (residues 1-124, Hutchinson and Mutopo, 1979). The N-terminal mutation in both variants may contribute to the reduced binding of bilirubin in this region.

The C-terminal mutation in albumin Redhill appears to involve an insertion of a short sequence, possibly localised in CNBr fragments 6 (447-548) or 7 (549-585). This difference was not resolved on acid/urea/Triton X-100 PAGE suggesting that the Triton binding properties were not affected by these additional residues.

The mutation is neither spontaneous nor does it lead to an alteration in the reading frame of the mRNA during translation. An insertion of base pairs into a gene in any number other than an exact multiple of three causes a shift in the reading frame, leading to a considerable alteration in the amino acid sequence following the position of insertion, which continues until a termination codon is reached. This has not happened in the albumin Redhill gene since the C-terminal and penultimate amino acid residues are identical to those of albumin A. Therefore, the mutation may have arisen *via* the unequal crossing over of homologues within the albumin gene prior to gene segregation. The extreme accuracy of most crossing over events depends upon the correct juxtaposition of the complementary homologues. However, the rare occurrence of misjoining between nearby regions of nucleotide homology within the gene can lead to a duplication (or deletion) of the sequence lying between the homologous regions. The mutation in albumin Redhill may have originated as a result of such an unequal crossover; the mismatching of short homologous nucleotide sequences positioned approximately ten to twelve residues apart within the albumin gene may have caused the duplication of the intervening sequence. The duplicate sequences would lie in tandem and account for the increase in molecular weight consistently observed in this region.

A similar mutation is observed in the β-chain of human haemoglobin. Two nearby regions of eight-nucleotide homology (residues 90-92 and 95-97) mispair resulting in either the deletion or duplication of residues 91-95. Human haemoglobin is very susceptible to mutation and many types have been identified. Mutations of this nature in an individual are not the result of new mutations in that person, rather they have been inherited from the previous generation and may have originated many generations in the past.

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ALBUMIN REDHILL, A HUMAN ALBUMIN VARIANT

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Summary Albumin Redhill, a variant human albumin with the same C-terminal amino acid as Albumin A but with arginine at the N-terminus has been isolated by chromatofocussing from the sera of an English family. Thus, Albumin Redhill contains two sites of mutation in its protein chain and is probably a proalbumin. The ability of Albumin Redhill to bind Ni(II) or Cu(II) ions is considerably less than that of Albumin A.

Keywords human albumin, variant, metal-binding, purification, proalbumin.

INTRODUCTION

Bisalbuminaemia, the presence of two electrophoretically distinct albumins in serum, is well documented. While numerous examples have been reported in humans [1], the structures and properties of these variant albumins are largely unknown. For example, the complete amino acid sequences of only five variant albumins are known [2-6] and one partial structure has been reported [7]. Abnormal albumins have been identified by their dye-binding properties [8]; unusual ligand binding properties of abnormal albumins have also been reported [9,10]. However, no systematic studies have been carried out on the relationship between the amino acid sequence of an albumin and its ligand-binding properties, mainly on account of difficulties in obtaining pure samples of abnormal albumins. We now wish to report initial observations on the structure and ligand-binding properties of an albumin which has been isolated from the sera of a mother and son living in the neighbourhood of Redhill, Surrey, U.K.

CASE REPORTS

CASE 1

This patient is a 69 year old woman with a clinical history of recurrent psychiatric illness, first presenting itself when she was 21 years of age. She had a hysterectomy and prolapse repair in 1973. In May 1976 she was shown by routine protein electrophoresis to have bisalbuminaemia. Since 1976 she has had many lengthy admissions to a psychiatric hospital on account of depression, although in between these episodes there have been periods of normality. Hypomanic attacks have also occurred during which she has not only talked incessantly but also has slept poorly. A high intake of alcohol has also occurred. Medication over the past 27 years has included amitriptyline, amylobarbitone, chlordiazepoxide, chlorpromazine, desipramine, haloperiodol, imipramine, nortriptyline, orphenadrine, perphenazine, thioridazine and trifluoperazine. She has also had courses of electroconvulsive therapy.

CASE 2

This patient is the 52 year old son of Case 1. He is mentally normal. He had a cholecystectomy in 1968 fc gall-stones, herpes in 1971, and a chest infection in 1972. At about the same time he had pain in the left leg; an X-ray of the spine and hips at that time, however, revealed only minor spondylitic changes. Recurrence of pain in the left leg was noted for a few weeks in 1982. The only medications which the patient has received are vitamin B compound, and amoxycillin for an infected finger.

The above reports summarise the main clinical data of the two patients. It is not implied, however, that any of the features quoted are necessarily related to the presence of Albumin Redhill; the documentation is provided simply to enable clinical comparison with any other cases which may come to light in the future.

MATERIALS AND METHODS

Blood was collected without addition of anticoagulant from both mother and son. The serum obtained by centrifugation was electrophoresed at pH 8.6 in 0.075 M sodium barbiturate buffer on a cellulose acetate membrane. After staining with Ponceau S, the presence of Albumin A was confirmed, but in addition, there was an approximately equal amount of another albumin with a relative mobility of 91 (alb A = 100).

Isolation

The two albumins were precipitated from the serum by means of addition of ammonium sulphate[11]. The albumin-containing fraction was desalted on a column of Sephadex G-100 (1.5 x 95 cm) using tris-HCl (0.01 M pH 7.0, containing 0.05 M NaCl) as eluting buffer. The albumin-containing fractions were pooled, dialysed against water and then lyophilised. The mixed albumins (25 mg) in histidine-HCl buffer (1 ml, 0.025 M, pH 6.2) were applied to a column (1 x 26 cm) of Polybuffer PBE 94 exchanger [Pharmacia (Great Britain) Ltd.] which had previously been equilibrated with the histidine-HCl buffer; the column was then eluted with Polybuffer 74 (diluted 1 to 8 with water and brought to pH 4.0 with 1 M HCl). The fractions which contained albumin eluted last and were pooled and then lyophilised; the residue was dissolved in the minimum amount of tris-HCl buffer (described above), and was then applied to a column of Sephadex G-100 (1.5 x 95 cm), which was, in turn, eluted with tris-HCl buffer. The fractions which contained Albumin Redhill were pooled, dialysed against water and lyophilised. Electrophoresis on cellulose acetate showed that the Albumin Redhill was contaminated with Albumin A. Pure Albumin Redhill was obtained after a second cycle of chromatofocussing. The molecular weight of Albumin Redhill was found to be 67,600, as determined by SDS-polyacrylamide gel electrophoresis[12]; Albumin A had a molecular weight of 65,400 by this technique. The isoelectric point of Albumin Redhill was 4.75 (Albumin A pI = 4.93). Immunodiffusion on microscope slides [13] against antiserum to human albumin (Miles Laboratories Ltd.) showed a single, strong precipitation line.

<u>Ligand-binding</u>

(a) <u>Nickel</u>

[^{63}Ni]-Nickel chloride (1 mCi) (Amersham International) was diluted to 1 ml with water and an aliquot (1 μl) was added to serum (30 μl) which had been treated with Chelex 100 (Biorad Laboratories); the mixture was incubated at 37^{o}C for 60 minutes. Electrophoresis of the mixture on cellulose acetate at 8 v/cm for 4 hours using sodium barbiturate buffer (0.075 M, pH 8.6) followed by autoradiography using LKB Ultrofilm (^{3}H) for 24 hours, showed that while Albumin A in the serum bound ^{63}Ni ions, Albumin Redhill did not.

(b) Palmitate

The experiment described above was repeated using ^{14}C-palmitate (Amersham International, 0.1 μCi, 1 μl) in place of [^{63}Ni]-nickel chloride. Autoradiography revealed that both Albumin A and Albumin Redhill bound palmitate to an approximately equal extent.

(c) Copper

This was determined on the defatted albumin using gel filtration [14] on a column (1.6 x 46.5 cm) of Sephadex G50 equilibrated with tris-HCl buffer (0.02 M, pH 8.0 containing 3 μg/ml Cu(II) ions). The column was eluted with the same buffer and the copper content of individual fractions determined by atomic absorption spectroscopy. Albumin A (5 mg) bound 5.48 μg of copper(II) ions under these conditions, whereas an albumin fraction (5 mg) containing approximately equal amounts of Albumin A and Albumin Redhill bound 3.92 μg of copper(II) ions. Hence, Albumin Redhill binds approximately 28% of the copper(II) ions that are bound by Albumin A.

C- and N-Terminal Amino Acids

The N-terminal amino acid of Albumin Redhill was shown, by the reaction with dansyl chloride followed by acid hydrolysis, to be arginine [15]. The C-terminal amino acid was shown, by partial hydrolysis of Albumin Redhill with carboxypeptidase Y, to be leucine [16].

Cleavage of Albumin Redhill by N-Chlorosuccinimide

Mixed Albumins A and Redhill were cleaved with N-chlorosuccinimide [17]. After the reaction was complete, the peptides were precipitated by the addition of 5 volumes of water, followed by 5 volumes of 25% trichloroacetic acid/1% 2-mercaptoethanol. The precipitated peptides were washed with acetone/0.2% HCl/1% 2-mercaptoethanol, followed by acetone/1% 2-mercaptoethanol and then dried *in vacuo*. SDS-Polyacrylamide electrophoresis[12] showed that Albumin Redhill had an N-terminal peptide, which migrated faster than that of Albumin A, while the C-terminal peptide migrated more slowly than that of Albumin A.

RESULTS AND DISCUSSION

Albumin Redhill has been isolated in pure form by chromatofocussing, a technique which has previously been used to purify Albumin Kashmir [18]. Albumin Redhill differs from other albumin variants which have been described so far, in that there appear to be two sites of mutation in the protein, rather than one. Cleavage of Albumin Redhill by N-chlorosuccinimide, at the single tryptophan residue trp214, reveals that both peptides thus produced differ from those obtained from Albumin A under identical conditions.

Albumin Redhill has arginine as its N-terminal amino acid, and could well be a proalbumin particularly as its molecular weight as determined by SDS-polyacrylamide gel electrophoresis is greater than that of Albumin A. Confirmation that there is a mutation near the N-terminus of Albumin A is provided from metal ion-binding studies. Albumin Redhill in serum does not bind [^{63}Ni]-nickel(II) ions and the binding of copper(II) ions to the defatted albumin is significantly less than that observed with Albumin A under the same conditions. The C-terminal amino acid of Albumin Redhill is leucine, as for Albumin A [10]. The variant albumin binds palmitate in the normal manner, indicating that the third binding domain of the albumin is unaffected. Albumin Redhill binds a number of ligands (bromophenol blue, bromocresol green, Ponceau S, Congo Red and bilirubin) less effectively than Albumin A [20], suggesting that the second mutation site may be near the binding domain for these ligands.

Albumin Redhill has a lower isoelectric point than Albumin A (4.75 vs 4.93), and Albumin Redhill is eluted after Albumin A on chromatofocussing, confirming that it has the lower pI. Albumin Redhill migrates more slowly than Albumin A on electrophoresis at pH 8.6; a contributory factor may bethe greater molecular weight of Albumin Redhill.

Further studies are in progress on Albumin Redhill, particularly the determination of its total amino acid sequence.

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